LYSOSOMAL ENZYME CHANGES AFTER ANTIGENIC STIMULATION

by

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Thesis presented for degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Medicine

October, 1970

To my wife, my son

and our friends

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DISCUSSION OF EXPERIMENTAL RESULTS

ACKNOWLEDGEMENTS

To Dr. D.M. Weir for his many hours of stimulating discussion and for his thoughtfullness to both myself and family, thank you. It was through these discussions that many of the suppositions put forth in this thesis were born and I hope I have acquired some of his talent towards scientific investigation.

To the individual members of the Immunology Unit who have been friends as well as colleagues I wish to express my sincere thanks. To Bill McBride, Ann McCracken, Janet Whyte, Dierdre Suckling, Jana Strambachova as well as the technical members of the Unit, my thanks for their co-operation and useful comments during my 3 year stay in Edinburgh.

The author also wishes to thank Dr. A. Stuart for his advice in the interpretation of histological sections; Dr. M.A.B. de Sousa and Professor R.G. White for their useful comments on autoradiography; Ian Flynn and Dr. I.B.R. Bowman of the Biochemistry Department for their helpful suggestions with respect to the enzyme assay system; Mr.J. Paul of the Medical Photography Department for his assistance in the preparation of the photographs (micro) used in this project, and finally to Mr. W. Lutz for valuable comments on the statistical significance of the results.

The author wishes to express his gratitude to the Medical Research Council for their financial support of this project during the past three years.

INTRODUCTION

Introduction

The subject matter of immunology is rapidly expanding to encompass areas beyond those of cell biology and medicine and is beginning to intrude into molecular biology and biochemistry. Much is known about the action of Lymphoid cell populations in the immune process but our knowledge of the molecular events underlying these general effects is in its infancy. The involvement of lysosomes in the immune response is still in its speculative stages of development. The following review of the literature, it is hoped, will give the reader insight into the current views of the workings of the lysosome as well as the functional roles which have been attributed to the organelle. The involvement of the lysosomes in the induction of tolerance will be discussed as will their proposed role in the initiation of the immune response.

This introductory review of the literature will be subdivided into five sections.

The first section will briefly deal with the involvement of the reticuloendothelial system in the induction of the immune response as well as its role in the defence mechanisms. There is a vast quantity of literature which has been compiled since Metchnikoff described his phagocytic cells and this section will necessarily be restricted to the literature pertaining to the involvement of the reticuloendothelial system in the immune response. Since there is an ever increasing amount of evidence which brings together the macrophage and lymphocyte as "partners" in this reaction, it is with this cell, the macrophage, that the review will begin.

In as much as the two cells have been connected in the mechanisms of the immune response, the section dealing with the lymphocytes

and lymphoid tissue follows the macrophage system. The lymphocyte has been assigned as the competent cell which eventually produces antibodies or becomes tolerant, as the case may be and the features and behaviour of these cells will be discussed in detail. The findings of several investigators suggest that the lymphocytes contain lysosomes with special features. These organelles seem to possess a functional role in the induction of the immune response and it will later be postulated that these organelles may also play a primary role in the induction of tolerance.

The localization of antigen and the cell types with which antigen reacts will be the next section of this review. Antigens which evade phagocytosis and remain extracellular may come into contact with the lymphoid cells and cause tolerance. With so much interest attached to the question of antigen processing many papers have been reported in following the pathways by tracing the fate of radioactive labeled antigens after injection.

Included in this introduction will be two sections which will deal with "some" of the current theories of both antibody production and the induction of tolerance.

As previously stated there has been compiled a vast array of information pertaining to the cytoplasmic organelles, the lysosomes. These organelles have been implicated with many biological as well as pathological phenomena and it is with this section that this review of the literature will end.

The main objective of this review of the literature is to enable the reader to become familiar with the interconnecting bridges which appear to exist between the macrophage, the lymphocyte, the induction of tolerance and finally to the small but apparently important: lysosome.

The Reticuloendothelial system (RES)

From the early studies of investigators such as Metchnikoff (1905); Pfeiffer and Marx (1898a) and Jaffe (1931), the existence of cells with phagocytic powers became known. These phagocytic cells classified together as the RES were found in the spleen, liver, lymph nodes and bone marrow.

Metchnikoff (1905) was the originator of the concept of the role given to the macrophage, as being the primary defensive cell in many species. This conclusion was drawn from the earlier work of Wyssokowitsch (1886) who had shown that bacteria in the circulation were not excreted in the urine or other secretions but were found by microscopic examination to be within the cytoplasm of the reticular cells which had presumably phagocytosed them.

It was Aschoff (1924) who gave the name "Reticuloendothelial System" to the group of cells with the ability to phagocytoze red blood cells, bacteria and other foreign particles. This system of cells is characterized by their common property of phagocytosis and comprises the reticular and endothelial cells of the spleen, the reticuloendothelial cells of the bone marrow and lymph nodes, the Kupffer cells of the liver and the monocytes of the circulating blood.

The earliest investigators believed that these cells which engulfed the foreign material were also the same cells responsible for the production of antibody. This belief was enhanced by the findings that overloading of the RES led to a diminished antibody response to a particular antigen.

Further support for this hypothesis came from the studies of Sabin (1939). She showed that the injection of an alum precipitated

azoprotein, which appeared coloured, entered into cytoplasmic vacuoles of the Kupffer cells in the liver where upon the colour vanished. When these cells later shed their cytoplasm, antibodies appeared in the circulation. Sabin postulated that these liver Kuppfer cells, which had phagocytosed the azoprotein, formed immunoglobulins from the engulfed material.

Later investigators however, found experimental evidence which did not implicate the macrophage as the antibody producing cell. Roberts and Dixon (1955) in transferring lymph node cells found that they were able to obtain a secondary response to bovine serum albumin (BSA) in irradiated rabbits, after challenge. They discuss the role of the macrophage, which made up 10% of the cells which were transferred; and were unable to attribute the antibody synthesis to these cells. The plasma cell population was very low but since 90% of the transferred cells were lymphocytes the possibility exists that these cells were responsible for the synthesis of anti-BSA antibodies. They concluded from this experiment that the major type of cell transferred was the lymphocyte and that it transformed directly to the plasma cell precursor without mitosis. In a later experiment Dixon, Weigle and Roberts (1957) using lymph node cells from primed rabbits, showed that if these cells were transferred to irradiated recipients and later challenged with BSA, there was a normal secondary response.

Evidence implicating the lymphocyte, as the antibody-producing cell, came from the earlier work of Harris and his co-workers (1945). In separate experiments they injected either typhoid antigen or sheep erythrocytes into rabbits. The lymphocytes were collected from the lymph and it was found that the cell extracts of these lymphocytes had a higher antibody titre than did the surrounding fluid. It was concluded that lymphocytes were instrumental in the formation of antibodies.

As previously mentioned it was Metchinkoff who first proposed that the macrophage was the cell which had an active role in the defence mechanisms of the body. The phagocytic capacity of these cells and the subsequent production of antibody are to a certain extent interdependent. "The micro-organisms which can be deeply injured by the direct action of the specific serum are few in number. In most cases this action is a feeble one and needs, for its completion, effective co-operation on the part of the phagocytes". These words were written by Metchnikoff in 1905, even then, it was apparent that macrophages played an important role in the immune response.

The co-operation between the macrophage and lymphocyte is a mutual one, for the presence of antibody in the circulation will enhance the phagocytic activity of the macrophage (Biozzi, et al., 1961). Mice were injected with small amounts of specific antiserum to Salmonella organisms and the subsequent clearance of I^{131} - labelled Salmonella from the blood was determined.

The co-operation between the macrophage and the immunocompetent cell has thus been established through the mediation of antibody. The appearance of antibody, during the initial stages of the immune response is accompanied by a marked increase in the rate of phagocytosis of the antigen (Uhr, et al., 1962).

Later in this text it will be shown how this co-operation is thought to take place. The role of the macrophage in the induction of the immune response will be discussed.

Lymphocytes and the Lymphoid Tissue

In 1810, Shobert, quoting the then "celebrated" Dr. Paley who was explaining the function of the spleen, said, "the spleen may be merely a stuffing, a sort of cushion to fill a vacancy or hollow, which unless occupied, would leave the package loose and unsteady".

Since these words were written we have come to realize the importance of both the lymphoid cells and the macrophages, their constant neighbours. The interrelationships between these two cell types and the induction of either tolerance or antibody production will be dealt with in later sections.

The source of the lymphocyte population has now been traced to both the thymus and bone marrow. This conclusion was reached from the studies of Miller (1967), who has shown that neonatally thymectomized mice when observed in their adult life have a much reduced output of thoracic duct lymphocytes, accompanied by a depletion of lymphocytes in areas of the lymphoid tissue. Since the animal does still maintain circulating lymphocyte levels, it follows that there must also be another source of these cells. It is thus very likely that the bone marrow also acts as a source of lymphocytes.

The relationship between the thymus-derived lymphocytes and those of the bone marrow has aroused a considerable amount of interest recently. Claman and his co-workers (1966) have shown that while neither a thymic-derived cell suspension nor bone marrow-derived cell suspension, could by themselves, elicit an immune response in lethally irradiated animals a mixed population of both these cell types could evoke a response when antigenically stimulated. The experiments of this group demonstrate a relationship between thymus- and bone marrow-derived lymphocytes in the response of irradiated

mice to sheep erythrocytes. This relationship has also been shown for other antigens. Using the model of Claman, Taylor (1969) has shown that when BSA is employed as the antigen once again an interdependence between thymic and bone marrow cells exists. It was found that both cell populations, present at concentrations of approximately 10^8 cells, were necessary in order to stimulate an immune response to BSA.

Gowans and McGregor (1965) have shown that it is the thymus-derived lymphocytes which are the long-lived cells. These recirculating cells were shown to respond to a given antigen by blast transformation and mitotic division but failed to go on to produce antibody; the bone marrow cells accept some sort of stimulus from the thymic lymphocytes and are the actual cells which synthesize antibody to a given antigen.

The lymphocyte series of cells may be subdivided into the following categories: small, medium and large. It is evident that lymphocytes morphologically similar may be functionally different. Gowans (1962) demonstrated that rat thoracic duct cell preparations could be freed of large lymphocytes by incubation at 37° C for several hours. The cell suspension obtained after this treatment contained more than 98% cells which were morphologically small lymphocytes.

Chromosome marker techniques, in mice, have shown that there exists a migratory stream of dividing cells from the bone marrow via the thymus to lymph nodes. This journey probably occupies several weeks, during which time the proliferating cells reach maturation (Ford, 1966). By contrast, the recirculation of the small lymphocytes from the blood into lymphoid tissue and back to the blood takes several hours. These cells are non-dividing and have an average life span of several weeks. The exchange of these small lymphocytes between the two regions, that is blood and lymphoid tissue, occurs

many times during the lifetime of a single cell.

The life span of rat small lymphocytes has been calculated to be in excess of 220 days (Robinson, et al., 1965). Through continuously injected tritiated thymidine it was shown that the small lymphocytes may be divided into two populations; a short-lived minority having a life span of less than two weeks and a long-lived majority with a potential life-span of many months.

Neonatal thymectomy, in mice of 5 to 6 weeks of age, leads to a 99% deficit in the thoracic duct output of small lymphocytes. The cells remaining in the circulating pool are lacking in their immunological competence (Miller, Mitchell and Weiss, 1967). If these same proceedings are carried out in rats, the deficit output of small lymphocytes is much less marked (Rieke, 1964).

Although most of the small lymphocytes produced in the thymus are destroyed locally without leaving the organ (Metcalf, 1967), there is evidence that a small proportion of the small lymphocytes are released into the blood stream (Weissman, 1967). Many of the small lymphocytes produced in the bone marrow are released into the circulation but it is not known whether or not these cells become part of the recirculating pool (Osmond and Everett, 1964). After the intravenous injection of isotope-labelled marrow cells, the small lymphocytes aggregate in the red pulp areas of the spleen. Although the actual number of cells localizing in these areas is small, the initial cells injected contain only a small percentage of cells which are classified as small lymphocytes and therefore the number of cells present in the red pulp of the spleen may be highly significant (Parrott, 1967). The actual origin of the recirculating lymphocytes is unknown but possibly the pool, in the case of the rat, contains both thymus - and marrow-derived small lymphocytes.

In the peripheral lymphoid tissue, lymphopoiesis appears to be largely dependant on antigenic stimulation. The areas most actively involved in proliferation, namely the germinal centres, arise as a result of stimulation by antigen, but here the evidence is rather conflicting. Germinal centres in the rat spleen do not contribute cells to the surrounding small lymphocyte population (Ellis, Gowans and Howard, 1968), however, upon primary stimulation by antigen, small lymphocytes capable of transferring immunological memory do appear in the recirculating pool (Gowans and Uhr, 1966) and evidence has been presented which suggests that the germinal centres are capable of memory cell production (Thorbecke, et al., 1967). It therefore seems likely that a proportion of the recirculating pool of cells is normally produced by the peripheral lymphoid tissue as a consequence of continuous antigenic stimulation from the environment.

There are probably some "lymphocytes" that have no concern with immunology, but from the immunological standpoint it can be reasonably stated that the circulating lymphocytes include: stem cells of a bone-marrow origin; progenitor cells as yet without experience of specific contact with antigen and finally, committed cells with potentialities to take part in antibody synthesis. Only lymphocytes in the small form are relevant here. These cells serve as carriers of information and each will be stimulated to proliferation only when it meets the appropriate circumstance usually antigen contact.

The small lymphocytes can produce severe graft-versus-host (GVH) reactions in suitable parent-into-F_1 combinations both in the rat (Gowans, et al., 1961) and in the mouse (Hildemann, 1964). The thoracic duct lymph represents the purest source of lymphocytes and since the contents of this duct discharge directly into the blood

stream, all the cell types found in the lymph must also be present at some time in the blood. Approximately 95% of the cells in the thoracic duct lymph are small lymphocytes (Gowans, 1959).

Examination of the ultrastructure of the thoracic duct cells has been made by Zucker-Franklin (1963). From these studies the general structure of the small lymphocyte has been observed. The nucleus is large and is usually indented deeply with the components of the cytoplasm surrounding the nucleus. Many of the lymphocytes examined revealed profiles of rough endoplasmic reticulum. This organelle was not thought to be present in the lymphocyte except under pathogenic conditions. Vacuoles, mitochondria as well as Golgi apparatus were all noted in the ultrastructural studies on the lymphocyte. From these studies, the presence of rough endoplasmic reticulum would therefore classify these cells as immature plasma cells possible precursor cells.

Using immunoflurescence with the appropriate specific antisera, van Furth (1964) found that cells reacting with sera against IgA or IgM could be recognized. Most of these cells were medium-sized cells. Small amounts of IgM were seen on small lymphocytes. The presence of the antibody-producing cells has been confirmed by Wesslen (1954) and Hallander and Danielsson (1962) by tissue cultures of thoracic duct lymph in vitro. More recently, the immuno-cyto-adherence test (Biozzi, et al., 1966) has demonstrated antibody on the surface of small and large lymphocytes. From the work of Sell (1967) it is likely that antigen interacting with antibody on the surface of the lymphocyte would bind complement through the exposed Fc portion of the antibody molecule resulting in the lysis of the cell. Sell (1967) has also shown that specific sheep antibody to

the gamma chains of rabbit IgG and the mu chains of rabbit IgM induced blast transformation of 100% of rabbit thoracic duct lymphocytes. In addition the small lymphocyte population of thoracic duct lymph can take part in the GVH reaction due to their ability to secrete antibody.*

Various cells have been shown to stem from the small lymphocyte. The rapidly proliferating small lymphocytes of the bone marrow may give rise to immunocytes (Harris and Ford, 1964); monocytes and macrophages (Volkman and Gowans, 1965) and mast cells (Goodman, 1964). Such stem cells are not immunocytes and in the absence of the thymus or its equivalent organ in other species, cannot confer immune capacity on an animal.

It was originally thought that the small lymphocyte was the end cell of the series. The experiments responsible for the general recognition that small lymphocytes are not necessarily end cells are undoubtedly those of Gowans (1962). Prior to these studies, large numbers of workers felt confident that small lymphocytes could give rise to descendant cells, this was the basis of the clonal selection theory in its first form (Burnet, 1959). The experiment of Gowans used the purest form of lymphocytes, thoracic duct cells from the rat, labelled them isotopically and used them to provoke a GVH reaction. With an adequate dose, 2×10^8 cells, a fatal GVH reaction occurred. Autoradiographs of tissues showed, after 24 hours, a large number of heavily labelled pyroninophil cells with large active nuclei in regions which 4 hours after injection of the cells had shown a large number of small lymphocytes. It was in these same areas of white pulp in spleen and primary follicles in the lymph nodes, that the damage brought on by the GVH was most evident.

* It should be noted that thymus-derived small lymphocytes are not thought to secrete antibody and therefore it is not intended that these cells be included with other cells of similar morphology.

Carstairs (1961) as well as Marshall and Roberts (1963) have shown that tissue cultures of small lymphocytes treated with phytohaemagglutinin (PHA) undergo mitosis and transformation. The general concensus of opinion is that the enlarged and dividing cells seen after PHA stimulation are equivalent to those obtained in the in vivo experiments of Gowans.

There is evidence that normal human lymphocytes can be stimulated to enlargement and mitosis by a variety of substances such as anti-leucocytic sera (Grasbeck, et al., 1963) or streptolysin S (Hirschhorn, et al., 1964). After lymphocytes have been stimulated by whichever agents are employed, there is an increase in the number of lysosomes within the cell (Allison and Mallucci, 1964; Hirschhorn, et al., 1964; and Hirschhorn, et al., 1965). Postulating the role of the lysosomes in the lymphocyte response to PHA, Hirschhorn and Hirschhorn (1965), proposed that the small lymphocyte unless stimulated has been shown to survive in vivo without division for a long period of time. These cells are apparently "repressed" in vivo, but when in culture with an appropriate stimulant, the cells become "derepressed". PHA has been shown to react with macroglobulins (Harris and Robson, 1963). The indirect action of PHA or other stimulating agents may be mediated through specific or non-specific binding of a subunit or precursor of gamma globulin, which, in the case of the small lymphocyte, acts as a "repressor" to prevent this cell from continuous specific metabolic activity. The binding of such a repressor to the PHA macromolecule would then be free to act intracellularly to produce a release of lysosomal enzymes. An alternative mechanism would be the release of the hydrolytic enzymes during the endocytosis of such macromolecular complexes.

Through the experiments of Gowans and McGregor, (1963), the depletion of lymphocytes by chronic drainage of the thoracic duct for several days reveals that depleted rats showed prolonged survival of first-set homografts of skin, coupled with a severe depression of the primary antibody response. The depletion of small lymphocytes brought about by X-irradiation leads to a depressed antibody response which may be remedied by injecting almost pure suspensions of small lymphocytes. These demonstrations of the importance of the size of the lymphocyte population strongly suggest that the effects of X-irradiation and cytotoxic drugs, allowing under appropriate conditions the development of tolerance, may, in most cases, be a simple result of the associated lymphocyte depletion. Perhaps the results are particularly important in underlining the fact that all tolerance is partial tolerance, and in showing once again that the population of small lymphocytes in the cortex of lymph nodes is as labile and mobile as the lymphocytes of the blood.

The Localization of Antigen

The introduction of a foreign substance into the body fluids of an animal leads to certain patterns of localization of the material. Early attempts sought to discover the cell type responsible for this localization of antigen in the hope that the underlying mechanisms of antibody production could be unravelled.

Thus colloidal carbon as well as various dyes were injected into experimental animals in the hope that the cell type involved would become known, but this technique proved too insensitive. The investigators were unable to establish the effect that the material had on the various cell types involved. It was not until isotope-labelled entigens became available that the localization of various antigens and the cell type involved became apparent.

McMaster and Kruse (1951) , Libby and Madison (1947) and Ingraham (1951) have all studied the fate of isotope-labelled antigen injected into animals. Using P^{32}-labelled tobacco-mosaic virus (TMV), Libby and Madison (1947) followed the fate of the antigen in the organs of mice. They examined the liver and spleen and found that they were both participating in the removal of the TMV from the circulation, moreover this activity persisted in these organs for long periods of time. The more detailed study by Ingraham (1951), likewise using an isotope-labelled antigen, gave similar results. In this experiment, Ingraham used S^{35}-sulfanilic acid-azo-bovine-gamma-globulin as his antigen in mice. Preparing autoradiographs of the liver, spleen and lymph nodes he found that a very small percentage of the antigen was retained for long periods of time. Animals

examined 200 days after the administration of the antigen were found to contain small amounts, still present in the liver and spleen.

It seems obvious that the antigen is retained during the antibody production phase and persists over a long period. It is not beyond the realms of possibility that a breakdown or removal of antigen from the site of antibody-formation is an essential prerequiste for the formation of the antibody itself (Johnston et al., 1955; Murray, 1968).

A number of serum albumins of comparable size have been shown to have relatively long-half-lives and are ingested by macrophages at a very slow rate. Nevertheless, when these same serum albumins were sufficiently heat-denatured, so as to diminish their solubility at the isoelectric point, they were rapidly ingested by the medullary macrophages (Benacerraf, et al., 1956). Lang and Ada (1967) proceeded to demonstrate that although these albumins had been denatured their antigenicity had not been altered. Using labelled heat-denatured human serum albumin (H.HSA), they followed the localization patterns in rats after hind-foot pad injections. The medullary macrophages of the popliteal lymph nodes showed a heavy localization as compared with the native human serum albumin (HSA) which gave weak grains in the autoradiographs studied. The H.HSA did not stimulate antibody production whereas the HSA gave weak, but positive results as determined by the Farr technique. If the H.HSA were mixed with anti-HSA there was the usual antigen-antibody complex formed, thus conclusions were drawn that the antigenicity of the H.HSA had not been destroyed through heating.

Although excellent immunogens, such as flagellae, show primary localization patterns in the germinal centres of unprimed animals,

other reasonably good immunogens such as haemocyanin do not. Such localization, attributed to the flagellae antigen, cannot be a necessary condition for the stimulation of a primary response. Such localization is also not a sufficient condition. Humphrey et al. (1967) primed mice with a hapten-protein conjugate (NIP-BGG) (Brownstone, Mitchison and Pitt-Rivers, 1966) which had been adsorbed onto alum. The binding power of the serum was measured for the hapten by the use of NIP-ovalbumin. After several weeks, when anti-NIP antibody was still present in the serum, the animals were given a further injection of soluble NIP-BGG which was labelled with I^{125}. Half the animals received the latter antigen the others received an injection of soluble I^{125} labelled NIP-HSA. Both antigens became rapidly and extensively localized in the germinal centres, by virtue of the anti-NIP present. However, several days later the animals which received NIP-HSA showed no rise in the anti-NIP titres whereas the mice which received NIP-BGG gave a large secondary response to the NIP hapten. Not only is this an illustration of the "carrier effect" but it also demonstrates that for a response to occur, the cells which have been primed to respond to a specific antigen must be present and that the localization patterns seen in the germinal centre have no special capacity to evoke them.

Medullary macrophages take up all antigens to some degree no matter the nature of the antigen. Thisis also true for tolerant animals but uptake of antigen tends to be somewhat greater when the recipients have been immunized actively or passively against the antigen. The uptake of antigens onto and into the reticular cells in the germinal centers occurs extensively with all antigens in primed on unprimed animals.

Nossal (1965) found that there was intense and early concentration of Salmonella antigens in the periphery of the germinal centres in rats. Using I^{125} labelled flagellae, the particulate form of the antigen, there was persistence of the antigen for approximately 3 weeks. If the soluble, I^{125}-labelled flagellin were used, antigen persisted for only 1 week. This localization appeared to be restricted to the macrophages which had invaded the area from the cortex. There followed an invasion of the label into the germinal centres with heavy follicular localization. After 2-3 days, the germinal centres were grossly enlarged with a large number of primitive cells appearing in the diffuse cortical sinuses. After 3 days, there were large increases in the numbers of plasmablasts in the medulla. However these cells had no label associated with them. Thus whether the antigen be particulate or soluble it has been shown that both forms will be localized eventually in the medullary macrophages as well as the primary follicles. The primary follicular localization appears to be associated with the dendritic macrophages of this area.

In the spleen, the localization of antigen is slightly different than in the lymph node in that the material which will eventually be trapped is brought to the organ via the blood stream rather than the lymph. Nossal (1967) in studying the localization of polymerized flagellin in rats found a similar picture to that expressed above in the lymph node. The white pulp of the spleen can be said to be the homologue of the lymph node cortex, for it contains both primary as well as secondary follicles. Throughout, there are diffusely scattered lymphocytes grouped around the central arteriole. The presence of labelled antigen which has been injected intraveneously

can be seen within minutes throughout the red pulp and accumulates in the marginal zone. Moving into the white pulp, the red pulp becomes clear of labelled antigen with the final arrival of antigen in the follicles themselves, the identical picture as seen in the lymph node is reached. The primary difference between the two organs is that in the lymph node there is often long-retention of the antigen in the cortex. The red pulp of the spleen however clears quite quickly and retention in this organ is predominantly in the follicular area.

The antigenic material which is localized in the follicles of the lymph nodes has been shown to retain its antigenicity for long periods of time, provided it is retained on the dendritic macrophages. Ada and Lang (1966) using flagellin in rats have shown that antigenicity may be retained to a greater degree when the material is taken up by the dendritic macrophages. In the follicles of the lymph nodes studied, the autoradiographs suggest that dendritic localization does not involve compartmentization of the antigen within the cytoplasmic vacuoles, as seen in the medullary macrophages. Therefore, the tertiary structure of the material may be retained for longer periods of time. These investigators also studied the localization patterns of haemocyanin as well as human serum albumin. The haemocyanin gave similar results to that of the flagellin in that both were associated with the medullary macrophage vacuoles. The HSA localized in the lymph nodes lightly with relatively little follicular localization. The general pattern is diffuse grains over the entire node. The antibody response to HSA was also low to absent, while the other antigens, flagellin and haemocyanin, which gave good medullary and dentritic localization, also gave good

response as determined by the Farr Technique. Boak, Kolsh and Mitchison (1969) also found retention of antigen in his studies using isotope-labelled, heat-denatured BSA. In these studies the antigen was retained within the peritoneal macrophages of cultured cells. This relates quite favourably to the in vivo retention studies. Approximately 90% of the original antigen which was engulfed was subsequently degraded within 2-4 hours. However, the remaining 10% was found to be held in "separate" storage vacuoles. Humphrey (1969) suggests that this material may be correlated with the enhanced immunogenicity of antigens that have been extracted from macrophages.

Thus in the spleen, antigens are first taken up by the macrophages of the red pulp and several days later the antigen begins to diffuse through the marginal sinus at the periphery of the white pulp. In both the lymph nodes and the spleen the injected material then localizes in the germinal centers of the primary follicles, more specifically on the dendritic macrophages. It has been suggested that the extent and speed of the accompanying dendritic localization is dependent either on the presence of naturally occuring opsonins or of specifically stimulated antibodies (Nossal, 1969).

In this brief review of the various studies undertaken to investigate the localization of antigen it can be seen that the importance of these studies must remain, somewhat, in dispute. From the studies examined, if a particulate antigen is employed in localization within the lymphatic tissue, there usually follows intense follicular localization which persists for several weeks. However, in the case of soluble antigens, such as the HSA antigen previously described, the localization pattern is less intense and distinct and tends to be diffuse throughout the organ.

Antibody Production

Evidence has already been cited indicating that a foreign material which enters the body fluids is rapidly phagocytosed by the macrophage. These materials are removed by the RES and are concentrated in the liver and spleen of the animal. There appears to be an inter-relationship between the phagocytic cells, which engulf the material, and the final formation of antibodies in the serum of the animal. Although the early investigators sought to explain the origin of antibody synthesis, antibodies in 1928 were still not proven to exist (Howell, 1928). It is now generally recognized that cells which are actively engaged in the process of phagocytosis are not actively involved in the actual synthesis of antibodies (Fishman, 1961).

One theory which attempts to explain the inter-relationship between the phagocytosed material and the actual synthesis of antibodies postulates that the great majority of the engulfed antigen is rapidly degraded within the lysosomes of the engulfing cell. However, a small proportion of this material remains unengulfed and it is this material which is taken up by the precursor cells for subsequent antibody production. There has been very little evidence for the existence of antigen within the precursor cells prior to antibody synthesis and this theory, while attractive remains to be proven.

A second theory involves the solubilization of the antigen by the phagocytic cell and the transference of a "message" to the precursor cell. It is interesting to note that the plasma cells, in the lymphoid organs, are often found in large clusters about the macrophages (Thiery, 1960).

The work of Fishman (1961) and Fishman and Adler (1964) provides the most striking evidence for the involvement of the macrophage in the immune response. They showed that the immune response involves a two-step process for the in vitro induction of antibody synthesis. The first step of the process involves the coming together of the antigen and macrophage. The antigen, T_2 phage, was mixed with rat peritoneal macrophages, ~~from rates~~, in vitro. The homogenized cell extracts were washed and incubated with lymph node cells in culture medium. These rat lymph node cells later gave indications of antibody synthesis. These results suggested that the initiating material which stimulated the lymph node cells was RNA since the addition of RNase to the tissue culture medium prevented antibody formation. Similar evidence for the participation of macrophages in the immune response comes from the studies of Askonas and Rhodes (1965). RNA preparations extracted from mouse peritoneal cells after the uptake of I^{131}-haemocyanin, in vitro, were found to be immunogenic in parental mice. The RNA contained I^{131} and had properties compatable with the presence of antigen or at least fragments of antigen. If RNA preparations were obtained from macrophages after the addition of I^{131}-haemocyanin, they too were found to be immunogenic, provided large amounts of antigen were incubated with the cells. In each instance, pre-treatment of RNA preparations with the enzyme RNase resulted in a diminished response. From these studies it seems apparent that macrophages do have the ability to complex antigen and RNA; this "super" antigen when mixed with lymphoid cells thus induces an immune response. The possibility also exists that the macrophage regulates the antigen concentration present in the circulation. This process would thus maintain the optimal antigen concentration in the circulating fluids over a long period of time.

An additional role of the macrophage may be the retention of antigen for long periods of time in order to supply the continuous flow of information necessary for a continued formation of antibody. delete

Therefore it appears that the phagocytic cells of the RES have a major role in the initiation of the immune response. However the nature of the mechanisms involved whereby the macrophage "informs" the lymphocyte of its needs to synthesize the appropriate, specific antibody remains unsolved.

As previously stated the continuous supply of immunologically competent cells, i.e. lymphocytes, may arise from the output of cells from the thymus (Miller, 1963). Taylor (1963) has suggested that these lymphocytes may require time to mature in the peripheral lymphoid organs.

As early as 1950, Marshall and White had stressed that the initiation of the immune response may not necessarily be attributed to one single cell type. Many situations may involve several cell types which eventually transform into plasma cells.

In the lymphocyte-macrophage and macrophage processing of some antigens, contact between cells is considered to be essential for the triggering of the reaction (Oppenheim, Hersh and Block, 1966). The in vitro studies carried out by these investigators revealed that column separated lymphocytes did not respond well to the antigen when two-fold dilutions of PHA and antileukocyte serum were added. Usually less than 1% of these cultures transformed. If, however, leukocyte-rich cultures were mixed with the human peripheral blood lymphocyte cultures, the response returned to normal. The data suggests that the presence of phagocytic cells is necessary as intermediaries for the secondary response of antigen-induced lymphocyte transformation, but that PHA and antileukocyte antisera act directly on the lymphocyte.

Induction of Tolerance

The specific suppression of formation of antibodies to a given antigen is a complex area of study in which the detailed mechanisms still remain obscure to-day.

As early as 1908 it was shown that x-irradiation of animals would suppress the development of serum precipitin formation if the radiation were given prior to the antigen (Benjamin and Sulka, 1908). Smith (1909) was the first to note that passive antibody administration led to the suppression of antibody formation; some years later, using the same experimental model, Glenny and Siedmersen (1921) found that antibody to diphtheria toxin, if passively administered led to the suppression of the primary response to diphtheria toxin.

Thus although suppression of the immune response was recognized at the turn of the century, little advancement was made during the first 50 years. The work of the above cited authors together with that of many other early investigators, laid the foundation on which the present day studies of immunolgocial tolerance continue.

Neonatal Tolerance

The use of neonates for the induction of tolerance was first described by Hanan and Oyama (1954) using protein antigens. The intraperitoneal injections of alum precipitated BSA during the first four weeks of life, followed by the administration thereafter of a booster dose of the same antigen, did not elicit an immune response as judged by the Arthus reactions. Dixon and Maurer (1955) induced tolerance to BSA by the subcutaneous injections of the antigen starting at birth and continuing the treatment for 3-4 months; the

induced tolerance persisted for 10-11 months after the induction treatment had been terminated. In a series of experiments using HSA in neonatal rabbits, Cinader and Dubert (1955, 1956) found that the injection of sulphanyl - HSA led to the formation of antibodies, in the tolerant animals, to the hapten but not the HSA. This gave an illustration as to the specificity of the induced tolerance in the adult rabbits.

The studies of Nossal and his co-workers were concerned with the induction of tolerance to soluble antigens. The flagellin of Salmonella adelaide if injected into rats at birth and thereafter given a twice weekly administration of the antigen resulted in a firm tolerance in the adult rats being established. (Nossal, Ada and Austin, 1965). If a single large dose of flagellin were given, tolerance was induced but its effect was not long lasting; numerous injections of flagella into neonatal rats produced tolerance but large amounts of flagella were required. If the flagellin antigen were labelled with I^{125}, the lymph nodes draining the site of injection showed no morphological changes between the normal and tolerant rats (Ada, Nossal and Pye, 1965). However, if flagellin and flagella were compared in neonatal and adult rats there were significant differences observed for these antigens (Nossal and Mitchell, 1966). In the neonates, the soluble flagellin revealed a diffuse pattern which included all the lymphoid tissue including the thymus. This material was not found to be phagocytosed even after the administration of passive antibody prior to the injection of the antigen. The adult rats, in contrast exhibited strong dendritic macrophage localization of the antigen (Nossal, Ada and Austin, 1964; Mitchell and Abbot, 1965).

Induction of Tolerance in Adults

The need to study tolerance mechanisms in neonates is no longer a necessary prerequist. It has been repeatedly shown that tolerance may be induced in adult animals by a single injection of antigen alone. Dietrich (1964) has shown this to be true in mice; Rowley (1965) in rats; and Dresser (1964) in rabbits.

The induction of tolerance in adult animals using large quantities of antigen stems directly from the studies of Felton and Ottinger (1942) who induced an unresponsive state using a polysaccharide antigen in mice. These experiments revealed that a long period of constant injection of the antigen resulted in a tolerant state being established.

The use of a large single injection of antigen to induce tolerance was explored by Dresser (1962). Adult mice were given a single injection of 150 mg BGG were completely tolerant. Sercarz and Coons (1963) demonstrated that tolerance which was induced by large doses of BSA was due to a lack of antibody forming cells rather than a "mopping up" of antibody by excess antigen. By means of a fluorescent antibody technique they could find no evidence of cells synthesizing antibody to BSA after the animals had been rendered tolerant to the antigen.

The work of Mitchison (1964) had a profound effect on the study of tolerance as we know it to-day. It was shown that the degree of tolerance which was induced to BSA was related to the amount of the antigen administered as well as the time course of the tolerance-inducing injections. Varying the amounts of BSA, the antigen was injected three times a week for 1-16 weeks. The result was two

zones of partial tolerance; the first zone, which has come to be known as high zone tolerance, was induced with doses of 5, 10 and 20 mg BSA administered for 8-14 weeks; the second zone or low zone tolerance was achieved by administration of 10 mg BSA for 1-4 weeks. The intermediate dose of antigen, that is lying between 10 and 5 mg BSA, gave normal antibody levels. The fact that tolerance and priming for subsequent antibody productions can occur simultaneously was clearly demonstrated by this work.

The work of Dresser (1962) focused much attention to the importance of the physical state of the antigen with respect to the induction of tolerance. In these experiments bovine gamma globulin (BGG) was found to induce tolerance in mice if the material were prepared by high speed centrifugation prior to injection. The aggregated pellet proved to be highly immunogenic whereas the soluble, aggregate-free material was able to induce tolerance. "Adjuvanticity" of the aggregated antigen, due to its molecular make-up seems to be the cause of the phenomenon, this "adjuvanticity" was removed by the high speed centrifugation of the material. Claman (1963) confirmed these findings of Dresser by the addition of endotoxin to a soluble BGG preparation. This mixture instead of inducing tolerance, ellicited an immune response. The conclusions were therefore drawn that the endotoxin imparted to the soluble material the "adjuvanticity", thus obtaining an immune response.

Biro and Garcia (1965) were able to induce tolerance to HGG by the injection of 5 mg of a centrifuged, aggregate-free preparation. If the aggregate-free preparation were heated at 63°C for 15 minutes, the HGG would elicit a normal immune response. This work also demonstrated that the induction period required to establish a tolerant state was greater than 24 hours and less than 7 days.

Termination of Tolerance

The termination of tolerance may be brought about by the cessation of administration of the antigen. The degree to which animals return to a normal state varies with the degree of tolerance induced and also with the various antigens employed.

Antibody production, in mice tolerant to BSA, was found to occur a few weeks after the circulating antigen could no longer be detected (Sercarz and Coons, 1963). This observation indicates that cells tend to return to their "former self" after the depletion of antigen. However, it has been shown that younger animals tend to return to normality more rapidly than do older animals which have been rendered tolerant (Mitchison, 1965). These results indicate that the younger animal has a more rapid turnover of its lymphoid cell population and the newly formed immunocompetent cells could not be rendered tolerant in this situation of antigen depletion. The antigen which did persist would sensitize these cells thus inducing antibody formation.

Tolerance may also be terminated by the injection of cross reacting antigens. By the injection of HSA into rabbits which had been made tolerant, neonatally, to BSA, Weigle (1961) was able to terminate the tolerant state. Further experimentation by Weigle (1964) revealed that although antibodies to BSA were recovered after treatment with HSA, these antibodies were directed against the shared antigenic determinants. Similar findings were reported by Mitchison (1964), in mice. However it was noted that the degree of reactivation was related to the degree of tolerance. In most of these studies there is a termination of partial tolerance rather than the termination of a completely tolerogenic state.

Mechanisms of Tolerance induction

There are many theories as to the detailed mechanisms of tolerance induction, as there are many investigators in this particular field of study.

If one applies minor variations to the selective theory of antibody production; there exists a population of cells which are capable of reacting with a suitable antigenic determinant. There may be one type of receptor per cell which would represent the clonal selection theory or there may be various receptors on these cells which would then support the subcellular selection hypothesis. The full activation of this cell type, no matter which theory was supported, requires another moiety which would be contributed by the macrophage. In the absence of macrophage participation direct binding of the unaltered antigen would occur with the cell's receptor. This combination would block the cell from receiving the "activated" antigen which had been processed by the macrophage. It follows that this cell would thus be inhibited from the further stage of transformation, that being antibody production, and would remain in a tolerant state.

The involvement of the macrophage in the induction of the immune response gains support from the work of Fishman (1961). Irradiated rats were implanted with chambers which contained lymph node cells from normal animals plus macrophage-treated T_2 bacteriophage. The combination of these two entities resulted in an immune response. If however, the lymph-node cells were mixed with the T_2 phage alone, there was no such response. In a subsequent series of experiments, Fishman and Adler (1963) and Fishman (1969), evidence was obtained which showed that the extracted RNA from macrophages containing antigen was able to elicit a specific antibody response.

Askonas and Rhodes (1965) pointed out that the immunogenic function of the macrophages may in fact depend on the presentation of antigen in the vicinity of a immunocompetent cell or may require the processing of antigen by the macrophage prior to the induction of antibody. They followed the fate of isotope-labelled haemocyanin in the peritoneal macrophages of the mouse. Soluble haemocyanin does not give a good primary response in the mouse. Their experiments made use of the cell transfer technique into primed animals. Normal spleen cells were incubated with RNA extracts of macrophages exposed to the antigen, this extract material was contaminated with antigenic fragments and the response they obtained was not due to a new specific RNA. From this experiment emerged the finding that macrophage RNA with its attached antigen was more immunogenic than treatment with antigen alone. It was also noted that a small amount of antigen persisted for days suggesting some binding or protective mechanism. Similar findings have been reported by Gottlieb and Straus (1969) using T_2 bacteriophage.

In adult animals if RES activity is suppressed, tolerance may result after exposure to antigen (Gallily and Feldman, 1967). Using a Shigella antigen which was incubated with peritoneal macrophages, the mixture was injected into animals which had previously been X-irradiated with 550r. This radiation dose was found to have a much more marked effect on the macrophages than on the lymphocytes. The initial mixture of antigen and cells resulted in the production of an agglutinating antibody. If antigen were given to the irradiated animals, without the preincubation with macrophages, there was no response. This provided evidence for the possible role of the macrophage in the processing of antigen as a necessary prerequiste for the induction of antibody production to certain antigens.

If however the antigen bypasses the macrophage and comes into direct contact with the lymphocyte the induction of tolerance may occur. This hypothesis is supported by the work of Mitchison (1968). He transferred lymphocytes exposed to antigen (BSA or HSA) in vitro to X-irradiated hosts and examined their sera for antibody production after subsequent challenging. There was a decreased antigen binding capacity in the recipient's serum.

The conclusion drawn from this set of experiments suggests that the processing of antigen by macrophages result in an immune response whereas if the antigen comes into direct contact with the lymphocyte the induction of tolerance occurs.

Lysosomes

In 1898, Von Prowazek, while studying Paramecia noted around newly formed food vacuoles, tiny vacuoles which were prominent when stained with neutral red. Several years later, Nierenstein (1905) made similar observations to those of Von Prowazek but further noted that these tiny inclusions shed their contents into the food vacuoles. He proposed that the shedding of their contents into the food vacuoles, in this manner, was in part, a means of cellular digestion. Thus it was that the existence of these digestive vacuoles were first noted although the name lysosomes was not applied to the organelle until much later.

Biochemical studies of lysosomes

The discovery of these organelles, as we know them to-day, was an unexpected result of a biochemical investigation. The later demonstration of their cytological existence was a confirmation of earlier experimental evidence indicating their presence reported by Appelemans, Wattiaux and De Duve (1955). By the use of differential centrifugation these authors found that the enzyme, acid phosphatase, sedimented partly with the mitochondria and partly with the microsomes. Mitochondrial association was characterised by the oxygen uptake of cytochrome oxidase and the microsomes were characterized by their associated glucose - 6 - phosphatase. From the sedimentation characteristics of the enzyme activity the authors proposed the existence of granules smaller in size than the mitochondria but larger than the microsomes.

These studies were pursued by de Duve and his co-workers (1955) who studied the sedimentation characteristics of 13 enzymes.

Some of these enzymes were known to be valid markers for mitochondria and microsomes. They separated rat liver homogenates by differential centrifugation into nuclei, heavy mitochondria, light mitochondria, microsomes and supernatant fractions. The recovery in terms of enzyme activity were excellent. According to their distribution pattern, the enzymes fell into four groups. The first group comprised the mitochondria, of which cytochrome oxidase was one of the enzyme markers. The second group was the microsomal fraction with the glucose - 6 - phosphatase as the enzyme marker. The third group contained the enzymes acid phosphatase, ribonuclease, DNase, cathepsin and most of the B-glucuronidase. The name "lysosome" was proposed to encompass this group of hydrolases in order to emphasize their richness of hydrolytic enzyme content.

It is unlikely that all of the lysosomal hydrolases are contained within each lysosome since, for example, rat liver lysosomes do not appear to behave as an enzymically homogeneous particle (de Duve 1963).

The molecular makeup of the lysosomal membrane, is at present, not known. From the biochemical studies of many investigators, the structural basis of the lysosomal membrane has been thought to be similar to that of the erythrocyte membrane. Robertson (1964) demonstrated the presence, in almost all cell types studied, of a layer of material approximately 75 A^{o} wide. This limiting layer was found to be made up of two dark, i.e., heavy-metals adsorbing, layers each about 25 A^{o} wide and separated by a light layer (one which does not take up the electron dense stain) also some 25 A^{o} wide. Each set of a pair of dense layers which are separated by a

light layer is referred to as a "unit membrane". Unit membranes have been found as a general feature of many intracellular organelles. The endoplasmic reticulum is such a membrane. The structure of the unit membrane, as seen through the electron microscope, is evidently that of a highly ordered lamellar object.

Most of the chemical analysis of the cell membrane have been performed on fractions isolated from mammalian red blood cells. These cells are readily available in large quantities as a pure cell type and therefore various simple techniques may be employed which will result in the loss of the greater part of the cellular haemoglobin. Both light as well as electron microscopy may be used to confirm that the remaining ghost preparations are indeed cell membranes devoid of their haemoglobin (Ponder, 1955).

The chemical analysis of the isolated cell membranes reveal that for the most part they consist of: cholesterol, phospholipid and protein (van Deenen and de Gier, 1964).

Changes in the permeability of the plasma membrane have been studied by various means. Brandt and Freeman (1967), found that inducers of pinocytosis in amoeba cause as much as a 50-fold decrease in the electrical resistance of the plasma membrane prior to the formation of the typical tunnels and vacuoles. In this state, the thickness of the electron-transparent core or lamella of the unit membrane is at least twice as thick as that of the control. These changes are dependant on the initial concentration of calcium. These changes are reversed when the concentration of calcium in the external medium is increased. No direct evidence is available on factors affecting permeability of lysosome membranes and possible mechanisms can only be inferred from work on plasma membranes of the type described above.

Classification of Lysosomes

In order to reveal the heterogeneity of the lysosome population, Bowers and de Duve (1967), in a series of papers, have characterized the lysosomal populations in the rat spleen. It was shown that the splenic lysosomes have a greater mechanical sensitivity than do the liver organelles. Differential centrifugation of the rat spleen homogenates ~~in the rat spleen~~ revealed that the hydrolases were associated with lysosome-like particles. These particles were shown to belong to two possibly three, distinct groups. These groups are known as the L15, L19 and L30 groups, respectively. Each group is recognized by the density at which the lysosomes come to rest, i.e. the L15 group had a density of 1.15. The L19 group of lysosomes contained a full complement of all twelve hydrolytic enzymes known to be associated with the lysosomes. The L15 group contained only the cathepsin, acid phosphatase and B-glucuronidase enzymes. The rest of the lysosomal enzymes were either not present in sufficient quantities or absent all together. The L30 population of lysosomes, as did the L19 group, revealed all hydrolytic enzymes to be present. It has been suggested that the L15 population of lysosomes belonged to the lymphocytes (Bowers, 1968). As they lack a full array of the hydrolytic enzymes, it is possible that these lysosomes have some functional role in the partial breakdown of proteins. They are obviously not participating in the storage of foreign material and likewise are too ill-equipped to engage in the intra-cellular digestion, as do the L19 group. The L19 group of lysosomes with their full set of hydrolytic enzymes are now

thought to be associated with the macrophage. Hayashi (1967) using histochemical staining techniques on rat tissue found that of the enzymes studied, acid phosphatase and B-glucuronidase, only the macrophages seemed to reveal any stainability. Further evidence as to the heterogeneity of the lysosomes comes from this work for it was found also that the cells varying in origin also varied with respect to the intensity of enzyme staining observed.

To-day it is recognized that all cells, possibly with the exception of the erythrocytes, contain lysosomes (Allison, 1968)

Site of origin of lysosomes

The formation of lysosomes has caused much speculation in this field of investigation. It is known that protein synthesis in the cell cytoplasm occurs at the ribosomal level (Tso, 1962). Cowie and his co-workers (1961) have studied the hydrolytic enzyme synthesis at the ribosomal level.

It is thought that these hydrolases may pass from the ribosomal sites of synthesis to the lysosome either by formation of lysosomes directly from the endoplasmic reticulum or via the golgi apparatus.

Wolman and Weiner (1963) suggested that lysosomes are derived from the endoplasmic reticulum by a change of this structure into a "water-in-oil" like system in which the membrane is folded into a globular body. By this means, the enzymes normally situated on the outer surface of the membrane are enclosed in vacuoles founded by the invagination of the endo-plasmic reticulum.

Novikoff (et al., 1964) stress the importance of the golgi apparatus in the formation of lysosomes. By means of electron microscopy, using autoradiography, it could be shown that newly synthesized proteins were located in the rough endoplasmic reticulum.

These proteins were then transferred to the golgi complex and were eventually deposited in a dense granule. The presence of acid phosphatase activity in the golgi apparatus was further suggestive evidence for the formation of the hydrolytic enzyme "pouches" being formed at the golgi level.

Whether either or both these explanations are applicable to lysosome formation may depend on the cell type studied.

Cohn and Benson (1965) added more strength to the golgi involvement in lysosome formation in their studies with mouse peritoneal cells. When cultured in vitro there was cellular transformation of the "monocyte-like cells" to phagocytes. This change was accompanied by the synthesis of at least three acid hydrolases which were concentrated granules with properties of lysosomes. By skillful use of autoradiography, electron microscopy as well as cytochemical staining they have produced strong evidence that these enzymes follow the same pathway as proteins synthesized for extra-cellular secretion; namely, the label first appeared over the ergastoplasm, then over the golgi region and finally within pinocytic vacuoles.

The formation of the dense granule, with its army of potent enzymes, is now classified as a primary lysosome. Within the lysosome group, there was a marked distinction between the primary and secondary lysosomes. The latter group are sites of present or past digestive activity while the former group or "virgin" lysosomes, contain their array of enzymes and have never come into contact with foreign material to digest. According to the origin of the material being digested the secondary lysosomes may be

classified into autophagic or heterophagic lysosomes. It should be noted that to some extent this distinction is rather a formal one since the two lines converge rapidly and material of either origin may be digested simultaneously or successively by the same enzymes.

The bringing together of a primary lysosome with its enzyme contents and the phagocytic vacuole with its engulfed material has given rise to hypothesis after hypothesis. The generally accepted view has its basis in membrane coalescence: as the membranes of the two vacuoles meet, they become reorganised into a single, continuous membrane encompassing the two original organelles. The main feature of this phenomenon is that the membrane remains continuous which shields the cytoplasm from attack by the contents of the lysosomes. Although this process, itself, may be rapid and difficult to observe by means of electron micrographs, several investigators have shown this fusion of the two organelles in several cell types: namely in macrophages (North and MacKaness, 1963), and in L - strain fibroblasts (Gordon, Miller and Bensch, 1965).

Lysosomes and their functions:

The importance of lysosomes in the digestive processes of the phagocytic cells, has been suggested by the very nature of these enzymes when first discovered by de Duve (1955) and received early support from the studies of Novikoff (1961), who stressed the high activity of acid phosphatase in the Kupffer cells and other macrophages. Essner (1960) clearly demonstrated the presence of acid phosphatase in erythrophagocytes. The close relationship between lysosomes and cytoplasmic vacuoles containing engulfed material has been demonstrated in numerous studies which depended on the acid phosphatase reaction for the recognition of lysosomes. Substances have been used which by their physical properties were impervious to digestion to illustrate the role of the lysosome as a storage site.

Materials such as iron (Kent, et al., 1963); carbon (Meijer and Willinghagen, 1961) as well as dyes and drugs (Allison and Mallucci, 1964) have all been used in this connection with the invariable result that the storage site was identified as a lysosome.

There are many examples throughout the literature of certain agents, pharmacological, as well as toxins, which render the lysosomes more permeable in vitro, and have been shown to have similar effects in living animals. Weissmann (1967) in an extensive review on this subject has stressed that these agents which increase the permeability of the lysosome membrane are at the same time lethal to the cells involved. X- and ultraviolet irradiation disrupt lysosomes in vitro and in living cells (Weissmann and Dingle, 1965). Endotoxins have been shown to have a labilization effect on lysosomes in vivo, but this effect has not been shown to have the same result on isolated lysosomes of animals treated in vitro (Slater, Greenbaum and Wang, 1963).

As a converse to the above statements, there is now considerable evidence which suggests that drugs which tend to stabilize lysosomal membranes also reverse the effect of cell and tissue damage brought about by agents which labilize lysosomes. Cortisol, cortisone and chloroquinine protect lysosomes against damage by ultra-violet irradiation (Weissmann and Dingle, 1965), carbon tetrachloride as well as vitamin A (Janoff and Zweifach, 1963) and Streptolysin O (Weissmann, et al., 1963). It has also been shown that antihistamines and cholesterol have stabilising effects on the lysosomal membrane (de Duve, et al., 1962).

Thus from the evidence presented by the authors cited above it appears that lysosomes may have a functional role in the inflammatory processes and tissue damage. The diversity of the lysosome,

as seen to this point of the review, has greatly outdistanced the original "dreams" of Metchnikoff. The phagocytic granules observed were merely thought to relate to digestion.

Other activities of Lysosomes

Although the areas of digestion and tissue damage have been surveyed, other functions attributed to the action of the lysosomes remain.

The function of lysosomes in immunopathology has been postulated from the onset of findings that antigen may be taken up by phagocytic cells and stored in these cytoplasmic organelles. The foreign material enters the reticuloendothelial cells in phagosomes and after the fusion with the primary lysosome the material will be catabolized in the secondary organelle. Various antigens, such as I^{131}-labelled albumin (Mego and McQueen, 1965) and antigenic bacteriophage (Uhr and Weissmann, 1965, 1968), have been shown to be sequestered and degraded in lysosomes of phagocytic cells. It should be noted, that in their 1965 report, Uhr and Weissmann were able to recover $\emptyset$ X174 phage from lysosomes after the phage had been engulfed by the phagocytes. The phage eluted from the lysosome granules was still able to elicit plaque formation when tests were carried out. Not only was the $\emptyset$ X174 phage found to be associated with the large granular fraction of the organs tested in the rabbit, but if the lysosomes were disrupted at various times after the injection of the phage, it was also found that 48 hours after the administration of antigen, the phage was more immunogenic than 1 hour after antigen administration. If vitamin A, an agent which has been shown to cause lysosome membrane labilization, were administered to the test animals the result was a prolonged 19S response which

is antigen dependent. It follows that these findings support the hypothesis that the sequestration and proper degradation of antigen within lysosomes is a necessary step towards the events progressing to antibody synthesis. However, it is possible that the increased lability of the lysosome membranes, induced by Vitamin A, resulted in failure of the 7S responding cells to produce antibody due to interference of their synthetic abilities by lysosome enzymes or due to their destruction by released lysosomal enzymes.

The effects of antibody on the lysosome have been studied by Dumonde and his co-workers (1965). By employing histochemical techniques the action of antibody plus complement were studied in ascites tumor cells. The results of this study suggest that the action of antibody and complement at the cell membrane surface leads to an activation of the lysosomal enzymes. In the presence of complement, antibody appears to enter the cell leading to labilization of the lysosomes. The labilization of the lysosomal membranes is suggested as being the causative effect of the accompanied cytoplasmic damage. While these tests were carried out in vitro, there is little reason why it may not be assumed that the same mechanisms operate in the in vivo situation.

If human peripheral blood lymphocytes are cultured in the presence of phytohemagglutinin (PHA) there follows a considerable increase in the number of lysosomes seen in the cytoplasm. This was found in the study by Hirchhorn, and her co-workers (1965). The observed increase was seen with both light microscopy as well as electron microscopy and the histochemically stained granules by both these observations were similar. The use of antigen stimulated lymphocytes follows a pattern similar to that of cells stimulated by PHA (Hirschhorn, et al., 1965). In this experiment

tuberculin-sensitive lymphocytes were cultured in the presence of tuberculin, and although only 20% of the cells transformed as judged by acid phosphatase increases there were, none the less, considerably more granules compared to the controls which contained the usual 2-3 granules. The possibility that lysosomes may sometimes let through large molecules, whether accidently or under special circumstances, must be entertained in light of the findings in antigen treated cells. Some intracellular proteins can gain access to the cytoplasm, as shown for instance by the cellular damage of ribonuclease (Brachet, 1957). Unless they enter directly through the cell membrane, their subsequent discharge from lysosomes must be assumed. A similar phenomenon must be postulated for antigenic proteins, especially if their immunogenic activity depends on partial hydrolytic breakdown by lysosomal enzymes. In the case of the lymphocyte lysosomes, which have a density of 1.15, there the full complement of hydrolases are not present in comparison with the macrophages (Bowers and de Duve, 1967). Since antigen-transformed lymphocytes, newly rich in lysosomes, have been observed both in vitro and in vivo, it is possible that they may mediate, in turn, the inflammatory responses to specific antigens (Weissmann, 1967).

It has been cited that agents, such as streptolysin S which stimulate cells in vitro, also bring about the labilization of the lysosomal membrane. Agents such as cortisone which stabilize the lysosomal membrane, appear to inhibit stimulation of cultures lymphocytes (Weissmann, 1964).

Due to the fact that the endocytic activity of many cells is a potent stimulus resulting in the formation of new lysosomes (de Duve and Wattiaux, 1966), the action of phytohemagglutinin on antigen-sensitive cells may result in the labilization of the lysosomes in the small lymphocyte.

Thus it can be seen that ever increasing "suggestive" evidence has gathered for the involvement of lysosomes in the immune response. If this evidence can be substantiated, the induction of tolerance, may also involve the lysosomes of the small lymphocyte.

The remaining chapters of this thesis will dwell on the lysosomal enzyme changes which occur after antigenic stimulation.

The use of Urographin gradients, in the separation of the large granular fraction into various subcellular peaks, lends itself as an excellent tool which may be applied to the study of tolerance. Animals which had been rendered tolerant to a given antigen would correspondingly portray a certain enzyme profile over a given density range. The lysosomal enzyme changes were studied after the administration of two forms of the same antigen. If enzyme changes did occur, following treatment with the foreign materials, one might postulate the involvement of lysosomes, with their entourage of enzymes, in the mechanisms of tolerance induction. Thus attempts were made to study the lysosomal enzyme changes following antigenic stimulation in mice. The cell types of the spleen were separated and enzyme changes in the lymphocyte population were examined.

Finally, attempts were made to determine the nature and dynamics of the enzyme changes in the lysosomes.

MATERIALS AND METHODS

SECTION

Materials and Methods

Animals used in this study

The experimental animals used throughout this study were the inbred strain of CBA mice. These animals were bred in the departmental animal house, the traffic light system of breeding being employed. The traffic light system of animal breeding involves three generations of progeny. The stock CBA mice were purchased from Carshalton and were classified as white card animals. The first, second and third generations were labelled as green, yellow and red respectively. Progeny from the original stock (white) were passed on to green cards; progeny from the green cards were passed on to the yellow cards and finally the progeny from the yellow cards were passed on to the red cards. Once reaching the red card stage, these animals became part of the experimental stock and were not used for further breeding lines. All new animals being brought into the animal house were placed in quarantine for two weeks to protect against infection being introduced in the main body of the stock mice. The experimental and stock animals were kept in separate rooms to further ensure the animal's safety from infection.

Animals of weights between 20 and 22 g were used in experimental groups, each group containing a maximum of 7 animals per cage.

A stock supply of normal NZW rabbits were also housed in a separate room, these animals were used to obtain normal rabbit serum (NRS) which was employed in several techniques used in this study.

Animal bleeding

Mice

All mice were bled via the retraorbital sinus using drawn glass pipettes. The pipettes were first rinsed in physiological saline

followed by a second rinse in heparin (100 units /ml.). The animals were held behind the head ensuring that the thumb and index finger held the head securely. The pipette was inserted into the marginal corner of the eye and the blood was collected once the sinus wall had been punctured. The pipettes were rinsed between each bleeding and the sera collected were placed into small disposable tubes. Incubation of the sera at 37° C for 20 minutes ensured clot formation and serum separation. The tubes were then placed at 4° C for 16 hours after which time the sera were centrifuged at 1000 R.P.M.(250 G) for 10 minutes. Using Pasteur pipettes the serum from each tube was carefully removed and these samples were then labelled and stored at -20° C until required.

Rabbits

Normal rabbit serum was obtained by means of cardiac puncture. The animals were held by an assistant and stretched out ensuring that the animal was incapable of any movement. The thoracic wall was saturated with methanol (70%) prior to each bleeding. The 19 gauge needle was inserted between the 3rd and 4th rib of the animal, slightly to the right of the midline. Once the heart had been punctured the blood was collected quickly and 30 ml were removed. The essential feature of this method of bleeding was the speed, in that an animal was bled in less than 1 minute.

The blood was placed into universal bottles and was thereafter placed in a water bath at 37° C for 20 minutes. Each tube was then ringed to ensure that the clot was free to contract after being placed at 4° C for 16 hours. The serum was collected and stored at -20° C until required.

Preparation of antigens

Explanatory note

During the preparation of all forms of bovine serum albumin (BSA), haemocyanin and sheep red blood cells, care was taken to ensure that contamination was avoided. In the case of the BSA and haemocyanin stock solutions, these were kept at -20° C and during the preparation of these antigens care was also taken to avoid frothing of the suspensions. If this did occur, denaturation of the protein would result and the material was discarded.

Materials

1. Bovine serum albumin (Armour Pharmaceutical)(Cohn V, >95% 8).
2. Alhydrogel (1.3% Al_2O_3)
3. Keyhole Limpet haemocyanin (KLH) (Calbiochem Co. Ltd.)
4. Sheep erythrocytes
5. Physiological saline (0.85%)

Procedure

Native bovine serum albumin (NBSA)

In a volumetric flask a stock solution of 0.25 mg per ml was prepared by dissolving 25 mg of bovine serum albumin powder in 80 ml physiological saline. The contents were allowed to dissolve, care being taken not to allow frothing of the solution which would result in partial denaturation of the material. The volume of the preparation was adjusted to 100 ml. Aliquots of the mixture were dispensed into 10 ml. samples and these were kept at -20° C until required. The preparation of the heat-denatured material (H.BSA) was carried by heating 5.0 ml. of the stock (NBSA) solution at 56° C for 30 minutes. After the experimental animals were injected, the excess material was discarded.

Centrifuged bovine serum albumin (CBSA)

Stock solutions of NBSA were allowed to come to room temperature. Four ml. quantities were then centrifuged at 40,000 R.P.M. (1.36×10^5 G) in an MSE Superspeed 40, using the SR40 rotor. The tubes were allowed to spin for 60 minutes at which time the machine was allowed to come to rest, ensuring that the brake was in the off position. The tubes were removed and using a 1 ml. syringe the top 2 ml. of each tube were removed. The prepared CBSA was then used immediately, the animals being injected with the material within 10 minutes of its being removed from the centrifuge tubes.

Alum precipitated bovine serum albumin (ABSA)

Equal volumes of a 5 mg preparation of NBSA and aluminum hydroxide were mixed (6 ml./6ml.). The mixture was allowed to stand at room temperature for 1 hour and then kept at $4^{\circ}C$ for 16 hours. This was usually done overnight for convenience sake. The preparation was centrifuged at 1000 R.P.M. (250 G) for 10 minutes and the supernatant was discarded. The remaining white precipitate was resuspended in 6 ml. sterile physiological saline and a smooth suspension was prepared by passing the mixture through a 19 gauge needle several times to remove and break the large aggregates.

Keyhole Limpet Haemocvanin (KLH)

A stock solution containing 2 mg per ml. was prepared. From the 2 mg solution, a 1: 20 dilution was prepared using physiological saline as the diluent. This resulted in a stock solution which contained 100 ug haemocyanin. The dose used throughout the experiments was 20 ug per ml. per animal. For each intraperitoneal injection of this antigen the quantity of antigen administered was 0.2ml.

Sheep erythrocytes (sRBC)

Sheep blood was collected directly from the abattoir and stored in an equal volume of sterile Alsever's solution at 4° C, for one week. After this period 3.0 ml. of the blood was removed, and the sample was washed three times in 0.85% saline. The erythrocytes were centrifuged at 1000 R.P.M. (250 G) for ten minutes and the supernatant was discarded, in each case. An aliquot of 1.2 ml. was removed from the washed packed erythrocytes and resuspended in 100 ml. physiological saline. The cells were counted by means of a haemocytometer and the final number of erythrocytes was adjusted to contain 1.0×10^{8} red cells per ml.

Preparation of Isotope-labelled bovine serum albumin

Introduction

The route antigen travels once it has been injected into an animal can be followed by means of isotope labelling of the protein. This is accomplished by the oxidation of the tyrosine residues on the antigen. The oxidized iodine molecule is substituted by the isotope molecule predominantly in the meta position of the tryosine residues. The method employed was the Chloramine T technique as described by Hunter (1967). Using this method small quantities of antigen may be labelled without denaturation of the material in question.

Materials

1. Phosphate buffered aline (pH 7.2) (see appendix)
2. Carrier free sodium iodide (NaI^{131} or NaI^{125}) (Radiochemical Centre, amersham, Bucks.)
3. Potassium iodide
4. Chloramine T (sodium p-toluenesulphonchloramide) 50 mg/100 ml. distilled water
5. Bovine serum albumin solution (20 mg/ml.)

Procedure

All labelling procedures were carried out in the hot lab in order to prevent radioactive contamination. Lead sheilding was constructed to protect against radiation and the entire technique was carried out in a fume cupboard.

A bijou bottle containing 1 to 4 mC carrier free I^{131} or I^{125} was placed on a magnetic stirrer. The buffer, 0.2 ml., was added followed by the protein to be labelled, which in this case was 0.2 ml BSA. The mixture was allowed to stir for 1 minute after which time the chloramine T, 0.2 ml, was added and the solution was

allowed to mix for a further minute. The mixture was then transferred to a dialysis sac by means of a hypodermic syringe. The reaction bottle was washed four times with 1.0 ml. buffer. This too being transferred to the dialysis sac. The sac was then tied and the mixture was dialysed against the buffer for 3 days, with the buffer being changed every 12 hours. This changing of buffer ensured that the free iodine which was not bound would be removed. The contents of the dialysis sac were then collected, again by means of a hypodermic syringe and the extenction coefficient was examined in a Unicam SP500 spectrophotometer at 280 mu. By comparison with that obtained with a standard BSA preparation * the concentration of the protein and the concentration of the I^{131} or I^{125}-labelled BSA was calculated in ug N per ml.

Prior to the injection of the labelled protein, the test animals were placed on a 1% potassium iodide drinking solution. This procedure ensured that the localization patterns observed in the autoradiographic studies carried out were not due to deficient iodine supplies but rather due to the antigenic nature of the foreign material being introduced.

* The protein standard determinations were calculated by W.H.McBride employing the microKjeldahl protein estimation technique.

Processing of tissues for histological examination

Introduction

The histological examination of tissues after antigenic stimulation requires that the tissues be processed and embedded in paraffin with subsequent sectioning of the organs. Various processing techniques were available and after several pilot studies, the single embedding procedure was found to give the most adequate results.

Materials

1. Formal saline (10%)
2. Alcohol (30%, 50%, 70%, 95% and absolute ethanol)
3. Toluene
4. Paraffin (R.A. Lamb, 12 the Viaduct, Ealing Road, Alperton, Middlesex)

Procedure

The tissues, liver, spleen and lymph nodes, were removed from the animals and each tissue was placed into 20 ml. formal saline. The fixation of the tissues was allowed to proceed for 18 to 24 hours. Dehydration of the organs was carried out by passage of the material through ever increasing concentrations of alcohol. Toluene was then used to remove the alcohol and to clear the tissue. The tissues were then transferred to molten paraffin which was kept at 52° C, after which time each organ was embedded in paraffin blocks. These were allowed to solidify and were then trimmed into individual organ blocks. Chart I illustratesthe time course employed in the processing of the tissue.

CHART I

SINGLE EMBEDDING PROCESS

Reagent	time (hours)
formal saline (10%)	18 to 24
alcohol (30%)	1.0
alcohol (50%)	0.5
alcohol (70%)	0.5
alcohol (95%)	1.0
alcohol (absolute) I	0.5
alcohol (absolute) II	1.0
toluene I	0.5
toluene II	1.0
paraffin I	0.5
paraffin II	1.0

Autoradiographic studies of I^{131} or I^{125}-labelled BSA

Introduction

The autoradiographic technique operates on the principle that the disintegrating radioactive molecule will emit an energized particle. A nuclear emulsion which is sensitive to such particles, if layered over the tissue, will reveal the position of the isotope with respect to the cellular components of the tissue.

Materials

1. Nuclear emulsion (Ilford L.4 or K.5)
2. Developer (Ilford ID19)
3. Fixative (Ilford rapid fix)
4. Xylol
5. Alcohol (64%, 74%)

Procedure

For the injection of isotope-labelled antigen the animals were removed from the departmental animal house and placed in the specially equipped isotope laboratory. At the termination of an experiment the animals were sacrificed by cervical dislocation and the tissues to be examined were removed. Each tissue was placed in a large volume of fixative (formal saline) and prior to the embedding procedure (previously described), the organs were weighed and determinations as to the amount of radioactive material present were calculated by means of a Nuclear Enterprises scintillation counter. The amount of radioactivity /mg tissue was then calculated.

The processed tissues were sectioned by means of a Cambridge microtome (rocker) and the sections, 4 to 6 u, were obtained for each organ. The sections were then placed onto glass slides and

were floated with 50% alcohol. The floating sections were carefully allowed to slip off the slide onto the surface of a warm water bath. The temperature of the water was not crucial, but was maintained in the 45° C range. The sections upon reaching the water flattened and specially prepared glass slides were used to pick up the sections from the water. A drop of "tissue-tac" (Dade Reagents, Inc., Miami, Florida) was placed on each slide prior to the lifting of the sections to ensure the tissue not separating from the slide during the manipulations which would be carried out later. The sections were placed in a 55° C incubator for two hours during which time the sections became fixed to the glass slide. The slides were then deparaffinized by passage through two changes of xylol (5 minutes for each change), followed by two changes of alcohol, 74% then 64%. The slides were then placed in water and were washed for 5 minutes.

Application of the nuclear emulsion was carried out under darkroom conditions with a yellow safe light as the sole means of illumination. The emulsion was placed in a cut off plastic graduated cylinder and kept at a temperature of 50° C. The melted emulsion was mixed with an equal volume of water and the solution was allowed to equilibrate for several minutes. The slides were then passed through the emulsion and care was taken to remove all air bubbles which may have gathered on the slide surface. The emulsified slides were allowed to air dry and were then stored in a light proof box, at 4° C, for several weeks exposure.

After exposure, the slides were removed and developed, once again employing darkroom conditions. Ilford ID19 developer was used undiluted for 5 minutes. The slides were then washed in water briefly and then fixed for a further 5 minutes. After the slides

had been washed, the slides were stained with either haematoxylin and eosin or methyl green pyronin, thereafter the slides were dehydrated through alcohol and after returning to xylol, they were mounted with glass cover slips and DePeX.

Preparation of Urographin gradients

Explanatory note

In 1967, a series of papers by Williams and Ada revealed that the separation of the large granular fraction of tissue homogenates on density gradients of Urographin allowed for subcellular separation of the internal organelles of the liver, spleen and lymph nodes. It was found that sufficient separation could be achieved which would enable the investigators to study the lysosomal enzymes of the homogenate as well as other cytoplasmic enzymes.

Materials

1. Urographin (3,5-diacetylamino-2,4,6-triiodobenzoic acid) (60%) (Pharmethicals (London) Ltd.)
2. Sucrose (0.29M)

Procedure

Depending on the density required, varying proportions of the two reagents were mixed. Chart II illustrates the quantities of reagent used and the resultant density.

CHART II

Density	60% Urographin	0.29M sucrose
1.057	0.30	3.00
1.100	1.08	3.00
1.125	1.80	3.00
1.150	2.61	3.00
1.175	3.00	2.25
1.200	3.00	1.50
1.250	3.00	0.50

The gradients were either prepared as those having a density range of 1.057 to 1.175 or those with a density range of 1.125 to 1.250.

Ensuring that each solution was thoroughly mixed, beginning with the 5th or bottom fraction, in each case, 0.9 ml. samples were layered into 5.0 ml. MSE Superspeed centrifuge tubes. Once completing the final layering (1.057), the gradients were allowed to equilibrate at 4° C for several hours prior to use. Care was taken in not disturbing the equilibrating gradients.

Preparation of the large granular fraction

Explanatory note

The preparation of the large granular fraction (LG) yields a variety of cytoplasmic components. This mixture of "cellular debris" consists of lysosomes, mitochondria, microsomes, nuclei as well as fragments of cell wall. Although this heterogeneous mixture of cellular components exists in the homogenate, nonetheless, through the employment of differential centrifugation on gradients of Urographin, it has been shown that separation of the components can be established. Williams and Ada (1967) have shown that through the use of these techniques the separation of mitochondria, lysosomes and microsomes can be achieved. Using enzyme markers, each enzyme was found to lie in a different density range, thus revealing the difference in organelles within that range. This finding was a confirmation of the original investigation carried out by de Duve and his associates (1955).

Materials

1. 0.29M sucrose

Procedure

The animal tissue being examined was removed and placed in 0.5 ml sucrose (0.29M). Immediately following removal of the tissue, the organs were prepared for homogenization. This was carried out on a mechanical homogenizer using a fresh volume of sucrose (0.5 ml.). The homogenizing tube was of pyrex with a specially fitted teflon grinder. After homogenizing the tissues for 5 to 10 seconds, the entire contents were diluted in 9.5 ml. sucrose, and were then transferred to the AR 40 rotor of the MSE Superspeed 40. The

initial separation was achieved by centrifugation 8,000 R.P.M. (4×10^3 G) for 10 minutes at 4° C. This force yielded an initial residue which was in turn discarded. The supernatant was removed to fresh tubes and subjected to a further centrifugation of 16,000 R.P.M. (2.1×10^4 G) for 10 minutes. The results of this final force was a small pellet which was the large granular fraction (LG). After removal of the supernatant, the remaining pellet was gently resuspended in 0.25 ml. sucrose.

The LG fraction was carefully layered onto the previously prepared gradients and the now entire gradient was centrifuged at 37,000 R.P.M. (1.14×10^5 G) for 16 hours. The rotor used for this final procedure was the MSE SR40. For convenience, these gradients were usually allowed to spin overnight. The next morning, the centrifuge tubes were removed and using a 1.0 ml. syringe, the various fractions were removed, 0.9 ml., beginning with the top fraction which had a density of 1.057. Aliquots were transferred to labelled Wasserman tubed to await the subsequent enzyme assay.

Freezing and thawing of the large granular fraction

Explanatory note

Lysosomes, being sac-like cytoplasmic organelles, reveal a structure-linked latency which may be overcome if the membrane bag is disrupted. One of the easiest methods employable is that of freezing and thawing. The result is the release of the internal enzymes which are thus more readily available to the substrate.

Materials

(as in the preparation of the large granular fraction, previously described)

Procedure

The same procedure was followed for the preparation of the LG fraction. Prior to placing the final homogenate onto the gradient, the mixture was placed in the deep freeze (-25° C) for 15 minutes. The frozen sample was allowed to melt at room temperature thereafter being frozen again. The twice frozen and thawed large granular fraction was placed on top of the gradient and the remainder of the procedure was followed as previously described.

Acid Phosphatase enzyme assay

Introduction

Several substrates may be employed to assay acid phosphatase. The p-nitrophenyl phosphate was shown to give a satisfactory standard enzyme curve and was thus employed. The fractions collected from the gradient were not disrupted, by the various mechanical means available, for any enzyme changes which occur would be compared, in later experiments, with the disrupted gradient fractions.

Materials

1. Acetate buffer 0.4M, pH 5.0 (see appendix)
2. p-nitrophenyl phosphate (substrate obtained from Sigma Chemical Co.)
3. Ethanol
4. Sodium hydroxide, 0.25M
5. Gradient fractions (enzyme)

Procedure

The freshly collected fractions from the density gradient were prepared in order to assay the amount of acid phosphatase present.

Each reaction tube received 0.1 ml acetate buffer (0.4M, pH 5.0). This was followed by the addition of the substrate in a measured volume of 0.05 ml. Finally, 0.35 ml of the fraction to be tested was added. This entire reaction mixture of 0.5 ml. was then incubated at 37°C for 30 minutes. To terminate the reaction, 2.0 ml. ethanol was added to each tube. The released p-nitrophenol was obtained by the addition of 0.2 ml. 0.25M sodium hydroxide.* The released p-nitrophenol was read on the Unicam SP500 spectrophotometer at an optical density of 410 mu. The reaction mixture employed by Williams and Ada (1967) was used throughout all enzyme assay procedures.

* The addition of the alkali strips the hydrogen from the hydroxyl group on the benzene ring thus changing the resonance of the structure. This resonance change results in the color changes observed.

Glass bead column separation of splenic lymphocytes

Explanatory note

A wide variety of different cell types populate the spleen. Cells which are in the process of producing antibody are morphologically as well as chemically different from cells which are in a dormant state. Separation of the dormant and antibody-producing cells is possible through a technique developed by Plotz and Talal (1967). Using the chemical changes which result through the synthesis of antibodies, separation is achieved by means of "stickiness" of the antibody-producing cells. The normal or dormant cells do not possess this characteristic, thus they pass through the glass bead column quickly. The sensitized cells, which have antibody on the cell surface, stick to the glass beads and are later eluted from the column by alterations in the environmental ions as well as pH. This technique enables the separation of lymphocytes to be achieved while preserving the integrity of the cell.

Materials

1. Hank's solution (H), (Ca^{++} and Mg^{++} omitted) (see appendix)
2. Hank's solution (HIP), (Ca^{++} and Mg^{++} present, plus 5% NRS) (see appendix)
3. Hank's solution (HIA), (Ca^{++} and Mg^{++} absent, plus 5% NRS, plus 0.001M EDTA)

Procedure

All columns prior to use were washed with concentrated hydrochloric acid followed by distilled water. Glass beads (1 or 2 mm) were used on all separation trials. The columns were dried in a

hot air oven and were ready for use after they had been filled to $\frac{3}{4}$ capacity with glass beads. The HIP buffer was added to the column ensuring that the level was above that of the glass beads. The column was then preincubated at 37° C for 1 hours prior to separation.

The spleens were removed and placed into 10.0 ml. H buffer. On a sintered glass homogenizer the spleen cells were then teased out until the remaining pulp of the organ was clear. The cell suspension was then passed through a 20 guage needle once, followed by a similar procedure through a 23 guage needle. The remaining debris was allowed to settle out and then 10.0 ml. H buffered cells was mixed with an equal volume of HIP and incubated at 37° C for 20 minutes. The suspension was layered onto the pre-heated column of glass beads and allowed to incubate at room temperature for 10 minutes. The various fractions were collected at a flow rate of 5 to 8 ml. per minute. In most trials, 5 fractions were collected each containing 60 ml.

In all trials there were 3 volumes of HIP buffer followed by $3\frac{1}{2}$ volumes of HIA buffer, passed through the column. The HIA buffer, containing EDTA, removed the cells adherent to the glass beads. The final 30.0 ml. HIA buffer was withheld until the column had emptied, this was then carefully decanted down the side of the column. The final fraction was then collected.

Each fraction was centrifuged at 500 G for 10 minutes. The supernatant was discarded and the suspension was reconstituted with 5.0 ml. H buffer. Each fraction was counted in order to obtain the number of cells present and these were represented graphically. These cells were tested in various ways.

Additional note

To further ensure the removal of any macrophages present, the prepared spleen cell suspensions were passed through a column of leucopac (sterile cotton wool) prior to incubation on the column. Leucopac columns were prepared as follows: 50 ml. plastic syringes were packed (5 cm) with sterile cotton wool. The cell suspension was allowed to pass through the column after which time the column was washed once to obtain the maximum number of lymphocytes from the suspension. The macrophages which have the characteristic of being "sticky" remain in the column and become trapped within the cotton wool fibres. The collected cells were applied to the glass bead column after their number had been calculated.

The immunocytoadherance test (ICA)

Introductory note

As previously mentioned in the section dealing with the glass bead column separation of lymphocytes, cells actively engaged in the process of producing antibody are different from those cells which are in a dormant state. The production of antibody by these cells enables one to examine another attribute of "stickiness" of the cells. Since by definition antibody is specific to a given antigen, the mixing of the antigen with the cells synthesizing antibody will lead to the formation of "rosettes". This then is the basis of the immunocytoadherance test (Biozzi, et.al., 1966).

Materials

1. Phosphate buffered saline (PBS) pH 7.2, 0.15M (see appendix)
2. Tannic acid (0.1 mg tannic acid/ml. PBS)
3. BSA (6 mg/ml.)

Procedure

Preparation of sheep erythrocytes

A 2% suspension of sheep erythrocytes was washed three times in PBS. The erythrocyte suspension was mixed with 5.0 ml tannic acid and allowed to incubate at 37 ^{o} C for 15 minutes. The cells were then washed once in buffer and half the suspension was set aside, to provide a sample of cells which were uncoated / which would be tested for rosette formation. The remaining cells were incubated with 5.0 ml BSA and placed in a 37^{o} C water bath for 30 minutes. The cells were then washed three times and their numbers were adjusted to 2 x 10^{8} cells per ml.

Preparation of spleen cells

The spleens were removed from the test animals and were placed into Eagle's solution. The organs were initially cut into small pieces and were then gently homogenized using a loose fitting, ground-glass homogenizer. The cells were filtered through a metal sieve and allowed to stand for 10 minutes at room temperature to allow any large particles to settle out. After washing the cells three times in Eagle's solution, the cells were adjusted so as to contain 2×10^7 cells per ml.

The immunocytoadherance test

Equal volumes of spleen cells and tanned cells (0.5 ml/0.5 ml) were mixed in Wasserman tubes. Included in the test were the uncoated, BSA treated cells. All tubes were placed at 4° C for 16 hours. The cells, after incubation, were diluted (1: 5 or 1: 10) and the number of rosettes present were counted using a Neubauer haemocytometer. The final number of rosettes/1000 spleen cells was then calculated.

Ammonium sulfate precipitation test (Farr technique)

Introductory note

The Farr technique (Farr, 1958) is of prime importance, in the detection of antibodies, for it is a measure of the primary interaction between antigen and antibody and allows for the total assay of antibody present in a given antiserum. Due to the sensitivity of the test, antibody detection may occur with samples of relatively low activity.

The Farr technique depends on the differential solubility of antigen and antigen-antibody complexes in the presence of 50% saturated ammonium sulfate. If complexes of antigen and antibody are present, under these conditions, they are insoluble, and precipitated complex is an indication as well as a measure as to the amount of antibody present in the test serum. This data, in turn, reveals the antigen binding capacity of the serum and is referred to as the ABC of the test sample. The percentage of bound antigen is plotted against the log reciprocal of the antiserum dilution. From this graph, the antiserum dilution which would bind 33% of the labelled antigen can be determined. This value is then used to calculate the amount of antigen bound per ml. serum. It is expressed in ug N bound per ml. undiluted serum at a given concentration. This resultant is expressed as the antigen binding capacity of the serum at a 33% end point, and is referred to as ABC 33.

Materials

1. Borate buffer pH 8.4 (ionic strength 0.1) (see appendix)
2. Saturated ammonium sulfate (SAS) specific gravity 1.24

3. Normal rabbit serum (NRS) (diluted 1: 10 in borate buffer)
4. I^{131} or I^{125}-labelled BSA (in 1: 100 NRS in borate buffer containing 0.01 N/0.5 ml. labelled BSA)

Procedure

The antisera to be tested were initially diluted 1: 10 in borate buffer followed by further five fold dilutions in 1: 10 NRS. The contents of each tube were mixed by means of a mechanical mixer. The pipettes were rinsed three times in borate buffer between the diluting of each test serum to ensure no carry-over of antibody from one sera to the next. Once the dilutions had been prepared, each dilution was then transferred to labelled Wasserman tubes. Two control tubes were included with each trial. One control (1) was for the total amount of antigen to be added, while the other control (2) determined the non-specific binding of the antigen to the 1: 10 NRS.

The experimental tubes each received 0.5 ml. of their appropriate dilution of antiserum. Each tube then received 0.5 ml. of the labelled I^{131} or ^{125} BSA. The tubes were gently shaken, and incubated at 4° C for 16 hours. Following the 16 hour incubation, each tube received 1.0 ml. SAS delivered by automatic pipette followed by immediate mechanical mixing. The tubes were incubated in an ice bath at 4° C for 30 minutes, after which time they were centrifuged at 750 G for 10 minutes. The supernatant was decanted, leaving the white pellet, and each tube was blotted dry. Care was taken to pool all the discarded supernatants for radioactive disposal. Each tube was then counted on the gamma scintillation counter and the antigen binding capacity of the sera were calculated. The calculation procedure was as follows:

1. Percentage of antigen added which is specifically bound/dilution.

$$= \frac{\text{COUNTS (EXPERIMENTAL)} - \text{COUNTS (CONTROL 2)}}{\text{CONTROL 1} - \text{CONTROL 2}} \quad (100)$$

2. The percentage of antigen bound/dilution was plotted against the log reciprocal of the antiserum dilution.

3. From the resulting graph, the antiserum dilution at the end point of 33% was observed. This point has been shown to be the most applicable for the comparison of antisera.

4. Finally, the antigen binding capacity (ABC) was calculated.

ABC = reciprocal of 33% binding (2)(.33)(0.01 gamma N)

(0.01 gamma N being the amount of antigen added)

Histological staining techniques

Explanatory note

Since most tissues do not retain enough color after they have been processed, in order that they and their components be made visible under the bright field microscope, it is necessary to add color to the tissues by means of stains.

One of the most general stains available is the haematoxylin-eosin stain. Using this stain the cell nuclei emerge as a deep blue, while the other elements take on a red pigment.

Special staining methods are available which enable the examiner to identify specific changes in the composition of the tissues. To detect changes in the state of polymerization of the nucleic acid of the cells, the methyl green and pyronin stains will distinguish between polymerization of the nuclei acids, but not the acids themselves, this technique will stain highly polymerized acids with the methyl green. The low polymers of DNA and RNA will stain with the pyronin.

The enzyme acid phosphatase exhibits an optimal activity at pH below 7.0, usually between 3.8 and 6.0. In many tissues, acid phosphatase appears to be associated almost exclusively with the lysosomes so much so that acid phosphatase is coming to be considered a "marker" for them. There is great variation in this technique, as will be seen from the incubation periods of sample and substrate. However, whilst variation occurs, indications may be seen as to reflect the over all occurance of the lysosomes and their enzyme marker, acid phosphatase, in the tissue examined.

A. Haematoxylin-eosin stain

Materials

1. Haematoxylin (Gurr Ltd.)
2. Eosin Y, C.I.45480 (Gurr Ltd.)
3. Sodium carbonate
4. Alcohol (varying concentrations)

Preparation of stains

Haematoxylin

Adding 1 g haematoxylin to 1 liter distilled water, the mixture was heated gently. To the heated mixture was added 0.2 g sodium iodate and 50 g potassium alum ($KAl(SO_4)_2.12H_2O$). The solution was left overnight to dissolve and 1 g citric acid and 50 g chloralhydrate were then added. The prepared stain was allowed to ripen before use.

Eosin Y

Two stock solutions were prepared. The first was a 5% eosin solution in distilled water. The second solution was prepared using ethanol, 2% eosin being required. To make 100 ml. of the eosin stain; 66.6 ml. solution 1 were mixed with 33.3 ml. solution 2.

Staining procedure

The following chart (III) illustratesthe reagents used in the staining of sections with haematoxylin and eosin. After the slides had been deparaffinized through xylol, alcohols and water, the procedure continued to the staining of the tissue.

CHART III

PROCEDURE FOR STAINING TISSUE SECTIONS WITH HAEMATOXYLIN AND EOSIN

REAGENT	TIME
haematoxylin	2 to 6 minutes (after 1 minute the intensity of the stain was examined microscopically)
running water	3 to 5 minutes
sodium carbonate	1 minute (this solution of alkali causes the nuclei to turn blue)
running water	3 to 5 minutes
eosin	1 to 2 minutes

The slides were transferred back to water, rinsing gently, taking care not to over wash the sections due to the water solubility of the eosin.

The stained sections were dehydrated through alcohols to xylol and were mounted using DePeX. The microscopic examination of the slides results in the observation that the nuclei were stained deep blue, and the other cytoplasmic structures were varying from pink to red.

B. Methyl green pyronin stain

Materials

1. Methyl green C.I.42590 (Gurr Ltd.)
2. Pyronin Y (Gurr Ltd.)

Preparation of stains

Methyl green

Adding approximately 10 g methyl green to 200 ml. chloroform, the mixture was vigorously shaken. Using suction filtration, the chloroform was extracted, removing the methyl violet breakdown products of the methyl green. After three extractions the dye was dried and stored in a stoppered bottle. A stock solution of extracted methyl green was prepared by dissolving 2.0 g of the dye in 100.0 ml. distilled water.

Pyronin Y

A stock solution of pyronin Y was prepared by the addition of 2.0 g pyronin Y to 100 ml. distilled water.

Staining solution

The methyl green pyronin stain was prepared by mixing the following solutions:

methyl green (2%)	7.5 ml.
pyronin Y (2%)	12.5 ml.
distilled water	30.0 ml.

Staining procedure

The deparaffinized sections were stained in the staining solution (described above) for 20 minutes. The slides were blotted dry and dehydrated in n-butyl alcohol (two, 5 minute changes). The slides were cleared in acetone, acetone-xylol (v/v) and finally in xylol, after which they were mounted with DePeX.

C. Acid phosphatase stain

Materials

1. Sodium alpha naphthol phosphate (Sigma Chemical Co.)
2. Fast red Garnet
3. Acetic acid buffer pH 5.0 (see appendix)
4. Polyvinylpyrrolidone (PVP)

Preparation of stain

The acid phosphatase staining solution was prepared by mixing the reagents as follows:

50 mg sodium alpha naphthol phosphate
50 mg Fast Red Garnet
375 mg PVP
50 ml Acetic acid buffer

Procedure

Spleen cell suspensions were prepared by careful homogenization of the spleens in 0.5 ml. standard Eagles media (which contains penicillin and streptomycin). The concentrated suspensions were washed and the final volumes were brought to 5.0 ml. This preparation was then passed through a leucopac column (as previously described) and the cells were collected. Centrifugation of the spleen cells was carried out at 150 G for 7 minutes. The supernatant was decanted and the final volume was reconstituted to 1.0 ml. Standard smears of the spleen cell preparations were made and the cells were allowed to air dry. Fixation of the cells was achieved by 10 minutes incubation in a 1: 5 stock gluteraldehyde solution, at room temperature. The fixed cells were washed with distilled water and were then incubated at 37° C for 12 to 24 hours in the

acid phosphatase stain. The cells were again washed in distilled water and were then transferred to a concentrated alkali solution of sodium carbonate for 20 minutes. This stage of the staining procedure causes the nuclei of the cells to "blue-up". The slides were then washed in distilled water and allowed to air dry.

The acid phosphatase stain when observed under the bright field of the light microscope results in the granules of cells appearing as black grains. These granules, containing acid phosphatase, represent the lysosomal organelles of the cell.

EXPERIMENTAL RESULTS

Section (1) Acid phosphatase activity of 10^6 spleen cells after antigenic stimulation in CBA mice

Lysosomes have been implicated, as having a functional role, in almost every biological process. It was for this reason that changes in the lysosomal enzyme, acid phosphatase were studied, after antigenic stimulation.

Various antigens were studied. The first was sheep erythrocytes, which although complex, stimulate a good response and have been shown to be macrophage dependant. The second antigen was keyhole limpet haemocyanin (KLH) which has been shown to be an excellent immunogen, and does not require the presence of macrophages in the induction of the primary immune response. The third antigen studied was bovine serum albumin (BSA). This antigen is malable in that various physical forms of the material may be prepared with relatively little difficulty.

Standardization of the substrate, as well as the enzyme, was the initial phase of this study. The substrate chosen was p-nitrophenyl phosphate. The enzyme assay, described by Williams and Ada (1967), was employed. A pure preparation of acid phosphatase, 1 mg, was diluted in water and various concentrations of the enzyme were assayed. The reaction mixture contained 0.1 ml. acetate buffer (0.4M, pH 5.0); 0.05 ml. p-nitrophenyl phosphate (0.05M) and the enzyme, in a volume of 0.35 ml. This reaction was allowed to incubate at 37° C for 30 minutes after which time the reaction was stopped by the addition of 2.0 ml. ethanol. The release of p-nitrophenol was brought about following the addition of 0.2 ml. sodium hydroxide (0.25M) and each sample was read at an optical density of 410 mu. The results of the standard enzyme titration are shown in Figure 1. The graph remains linear to an optical density of 2.0, thereafter the line begins to tail off and is no longer linear. Having established the substrate concentration required for the subsequent assays, the enzyme levels after antigenic stimulation were studied.

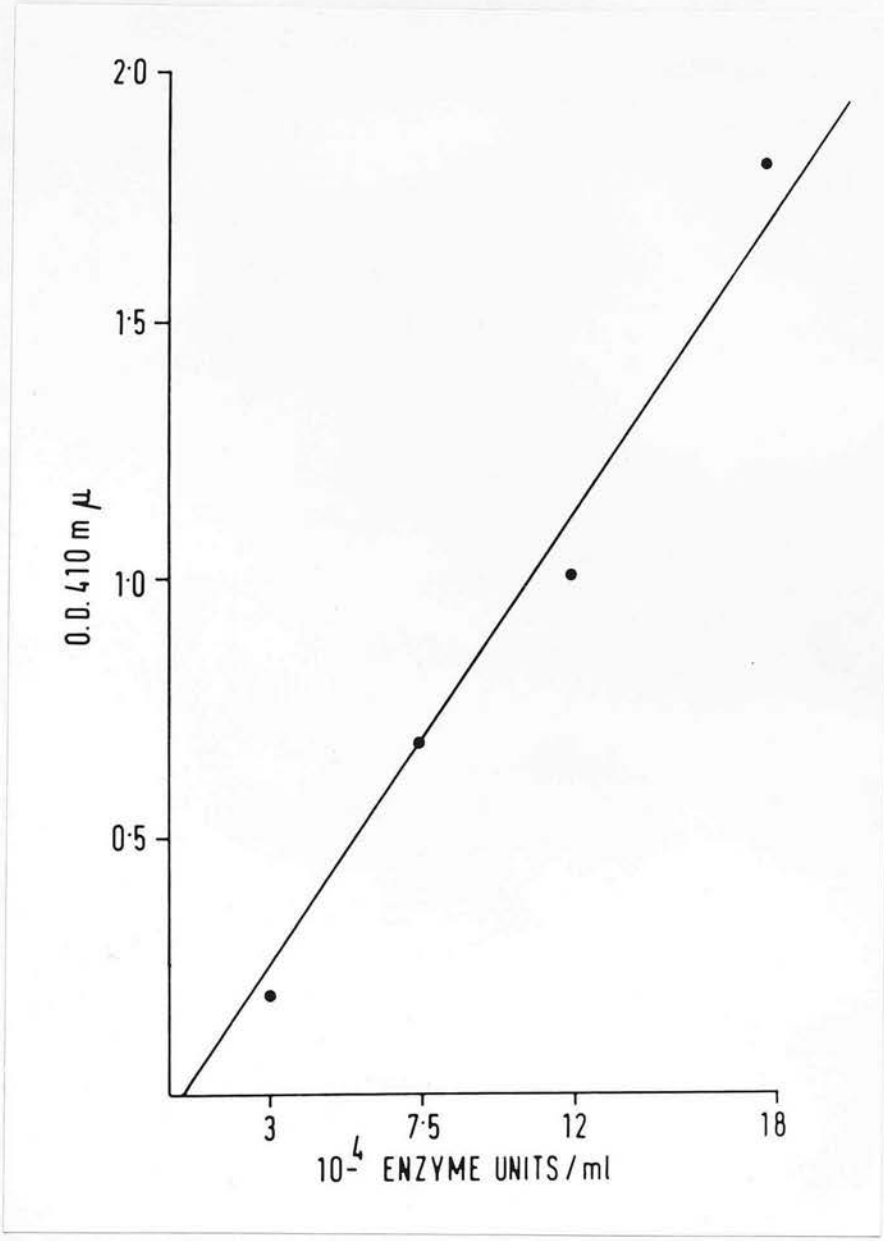


Figure 1. Standardization of the enzyme acid phosphatase. The enzyme activity is expressed as the number of umoles substrate consumed at 37° C for 30 minutes. The substrate was p-nitrophenyl phosphate.

The three antigenic materials used in this study were sheep erythrocytes (SRBC), keyhole limpet haemocyanin (KLH) and bovine serum albumin (BSA). The BSA was investigated in two different physical forms; one being heat-denatured and the other being a soluble, aggregate-free material. These two physical forms of BSA were employed since it has been repeatedly shown that the soluble, aggregate-free form of BSA is capable of induction of tolerance whereas the heat-denatured material has been shown to be more immunogenic than the former antigen.

Groups of CBA mice were injected with either of the following antigens: 0.05 mg heat-denatured BSA (H.BSA), 0.05 mg centrifuged BSA (C.BSA), 20 ug KLH or 2 x 10^8 sRBC. Forty-eight hours after the administration of the antigen the animals were sacrificed. The spleen of each animal was removed into 0.29M sucrose and following gentle homogenization, as outlined in the Materials and Methods section, the cell numbers were counted by means of a haemocytometer. The number of cells in each test sample was adjusted to 10^6 cells per ml. Each sample was then subjected to sonication for 10 seconds (amplitude 8 microns, peak to peak) and the enzyme assay was then carried out on the disrupted samples. The results of the enzyme assay are shown in Figure 2.

All the experimental groups, irrespective of the antigen they received, gave higher acid phosphatase readings than did the non-stimulated controls. Since it is difficult to determine, due to the inaccuracy of dilutions, the exact number of cells in each of the test systems employed it was decided to examine the large granular fraction of the whole spleen homogenate in order to observe the enzyme changes in a more accurate system. In the case of the cell counts, the exact dilution required to obtain the identical number of cells in each of the test systems was found to be too inaccurate for reliable results. Each antigen shown in Figure 2 represents the mean of 6 animals, the results of which may be seen in Table I.

TABLE I

Acid phosphatase activity of 10^6 spleen cells after antigenic stimulation

(i) C.BSA	(ii) H.BSA	(iii) Haemocyanin	(iv) SRBC	(v) Normal control
1.50	0.70	0.54	0.41	0.20
0.84	0.20	0.81	1.10	0.12
1.00	1.10	0.76	0.68	0.38
0.46	1.00	0.30	0.71	0.20
0.60	0.26	0.60	1.60	
0.73	0.35	0.29	0.20	
0.85*	0.60	0.55	0.78	0.22
±0.13**	±0.38	±0.13	±0.52	±0.04
0.05***	N.S.	0.05	0.05	

* Mean optical density 410 mμ

** Standard error (95% confidence limits).

*** The Rank Sum Test (see appendix) was performed on each set of figures comparing the experimental results (i-iv) to the normal, non-stimulated animal (v). A value of $P \leqslant 0.05$ was taken as significant.

If (ii) is not significant is (iv) really significant

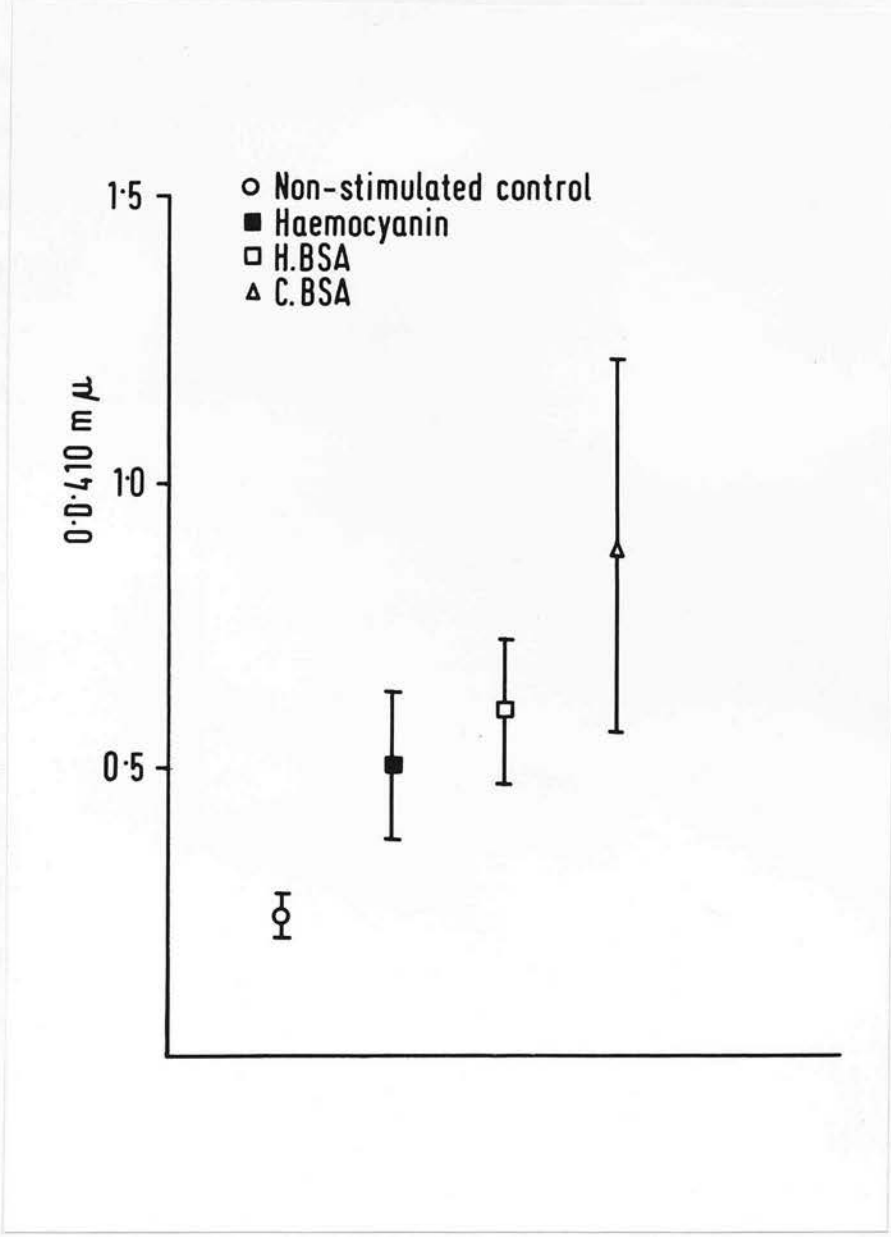


Figure 2. Acid phosphatase activity of 10^6 spleen cells after antigenic stimulation. Each point represents the mean of 6 animals.

Section (2) Acid phosphatase activity of the large granular fraction in spleen homogenates after antigenic stimulation

Differential centrifugation of splenic tissue allows for the recovery of many cytoplasmic organelles of the cell. The large granular (LG) fraction of the spleen consists of such organelles as microsomes, mitochondria and lysosomes, also nuclei and cell membrane fragments. This heterogeneous mixture was prepared as outlined in the Materials and Methods chapter and of prime interest were the lysosomes.

CBA mice were again injected with the various antigens described in the previous section. However, in this series of experiments, the use of the sheep erythrocytes as an antigenic form was omitted due to the complexity of the antigen. Prior to the assay of the enzyme acid phosphatase, the large granular fraction was put through two cycles of freezing and thawing. The enzyme determination, as shown in Figure 3, reveals the same pattern as previously shown in Figure 2. The antigen-treated animals all responded with higher acid phosphatase levels than did the controls. There were no differences observed in the enzyme values, whether the antigen be heat-denatured BSA (H.BSA) or the soluble, aggregate-free form of the antigen (C.BSA), both gave similar results. Included in this study was the haemocyanin which also gave an increase in the detectable amounts of acid phosphatase.

Each point represents the mean of 6 animals the results of which may be seen in Table II.

TABLE II

Total Acid phosphatase activity of the splenic disrupted large granular fraction after antigenic stimulation

(i) C.BSA	(ii) H.BSA	(iii) Haemocyanin	(iv) Normal
3.75	2.70	2.91	1.85
3.20	3.40	2.58	1.74
4.30	3.60	2.40	2.29
3.10	3.50	2.60	1.95
3.50	3.60		
3.60	3.00		
3.58*	3.30	2.62	1.96
±0.48**	±0.33	±0.14	±0.17
0.05***	0.05	0.05	

* Mean optical density 410 mμ

** Standard error (95% confidence limits)

*** Rank Sum Test

The value of p expressed is significant if $\leq$ 0.05

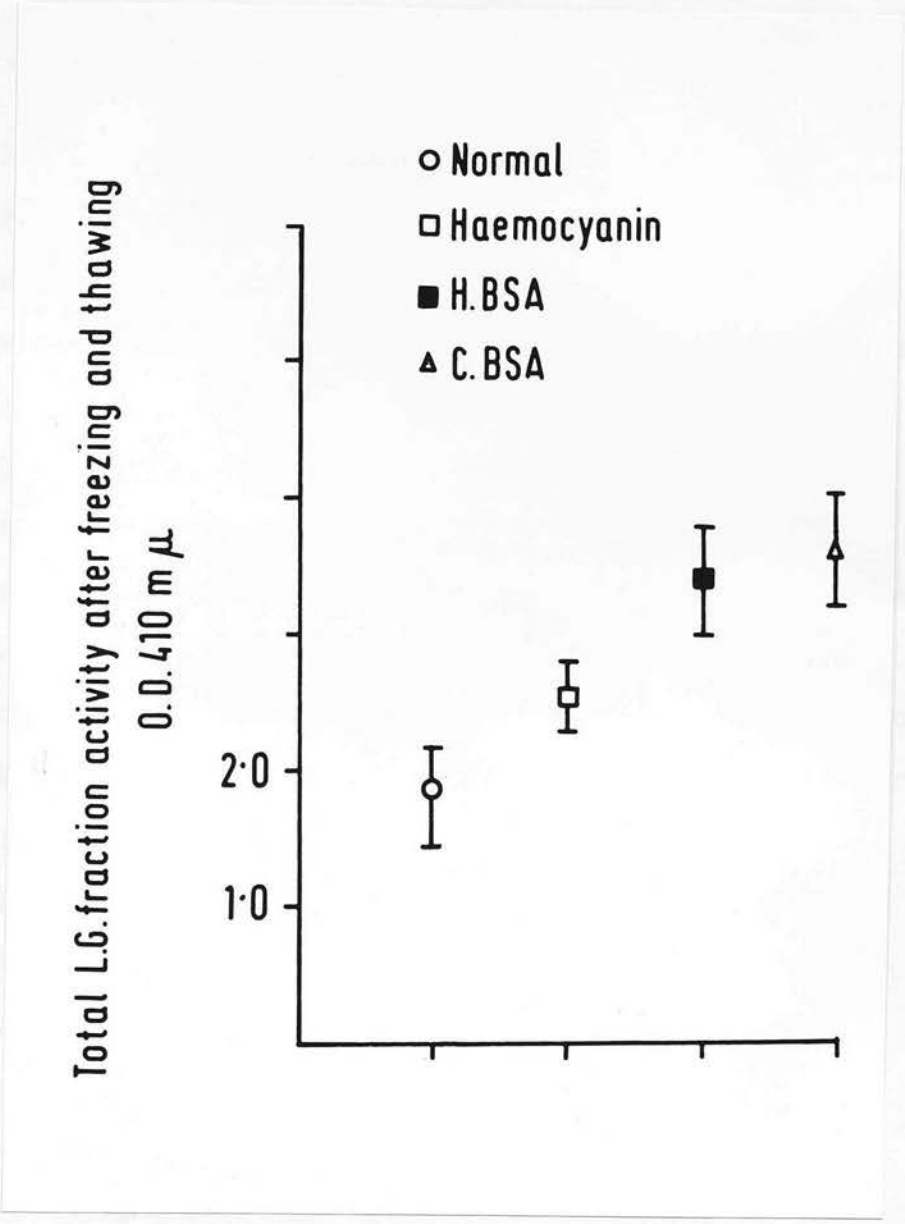


Figure 3. Acid phosphatase activity of the large granular fraction of the spleen after antigenic stimulation. Prior to the enzyme assay, the LG fraction was subjected to two cycles of freezing and thawing. Each point represents the mean of 6 animals.

Section (3) Acid phosphatase activity of the intact large granular fraction of the spleen homogenates after antigenic stimulation.

If the lysosomes were examined intact, without the freezing and thawing procedure, the results obtained after antigenic stimulation were markedly different from those observed in Figure 3.

Once again the antigens studied were heat-denatured BSA and the aggregate-free form of BSA. The observations obtained when intact lysosomes were assayed for the present of the enzyme acid phosphatase revealed a marked difference between the immunogenic antigen and the tolerogenic material. The same quantity of BSA but in two different physical forms gave opposing results. As seen in Figure 4, the C.BSA gave readings which were similar to the reading if the same antigen were administered and the lysosomes were disrupted. However, in the case of the H.BSA after antigenic stimulation, a significant depression in the quantities of acid phosphatase were detected. The C.BSA still gave results which were significantly higher than the non-stimulated controls. However, the immunogenic material, H.BSA, gave values which were less than the observed control levels.

Table III illustrates the differences obtained in the acid phosphatase activity which was detectable 48 hours after the injection of the antigen.

It should be noted that the aliquots used in the present assay were from the same pool used throughout the freeze/thaw studies. The samples were divided and were either assayed for intact or disrupted enzyme activity.

Soluble forms of antigen, as outlined in the introduction, have been shown to be capableof tolerance induction more

readily than the particulate, aggregated form of the antigen. The results on intact lysosomes reveal that C.BSA induces higher detectable enzyme levels than does the administration of H.BSA. The H.BSA brought about a depression in enzyme levels as compared to the control animals.

TABLE III

Total acid phosphatase activity of the intact splenic large granular fraction after antigenic stimulation

(i) C.BSA	(ii) H.BSA	(iii) Normal
5.04	0.41	1.09
3.15	1.30	1.59
3.59	.86	1.59
3.17	.86	1.95
3.35	.59	
2.63	1.01	
3.48*	0.86	1.55
±1.7 **	±0.25	±0.57
0.05***	0.05	

* Mean optical density 410 mμ

** Standard error (95% confidence limits)

*** Rank Sum Test, a value of $p \leqslant 0.05$ was taken as significant.

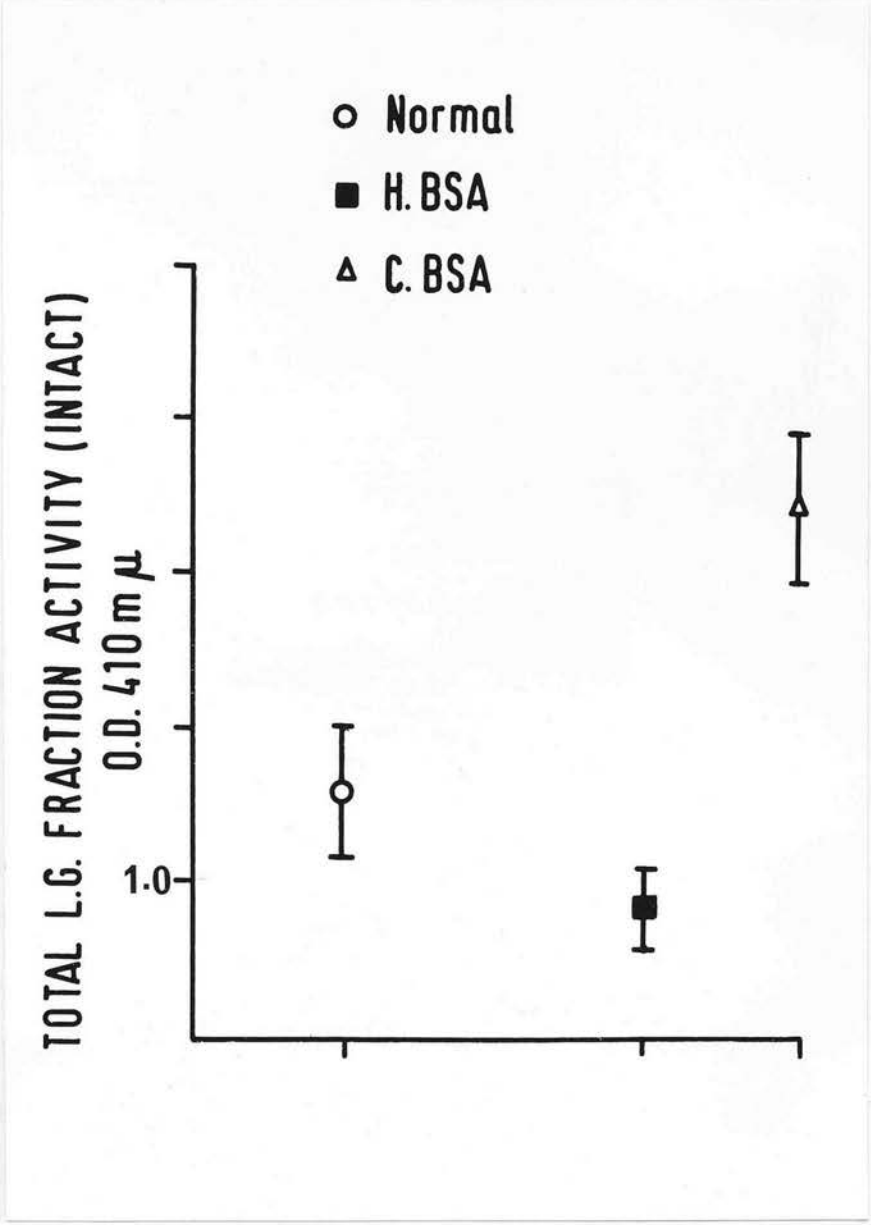


Figure 4. Acid phosphatase activity of the large granular fraction after antigenic stimulation, but prior to the disruption of the lysosomes.

Section (4) Density gradient centrifugation of the large granular fraction of normal CBA mouse liver and splee

The LG fraction of tissue homogenates contains various elements which are derived from the organ as a whole. Homogenization coupled with differential centrifugation yields a suspension which contains: fragments of cell membrane, mitochondria, microsomes as well as lysosomes (Williams and Ada, 1967). Changes in the acid phosphatase content of the LG fraction have been observed in the previous experiment. The distribution of the lysosomes was studied by means of density gradient centrifugation.

Normal, healthy CBA mice were sacrificed and following the removal of their spleens and livers into 0.29M sucrose, the large granular fraction was prepared as outlined in the Materials and Methods chapter. In each case the frontal lobe of the liver was removed to sucrose. The LG fraction was resuspended in 0.25 ml. 0.29M sucrose and carefully layered onto a density gradient of Urographin which had a density range of 1.057 to 1.175. The gradient was centrifuged at 37,000 R.P.M. ($1.14 \times 10^5 G$) for 16 hours at 4°C. Aliquots of the gradient were removed and each sample was assayed for the presence of acid phosphatase. The liver enzyme profile obtained for the normal, non-stimulated, animal is shown in Figure 5.

Figure 5 represents the liver enzyme activity over the density range of 1.057 to 1.175 and each point represents the mean of 6 animals. The peak enzyme activity is found in fraction 3, which has a density of 1.125. The maximum activity in the normal, control liver was a reading of 1.0, read at an optical density of 410 mu.

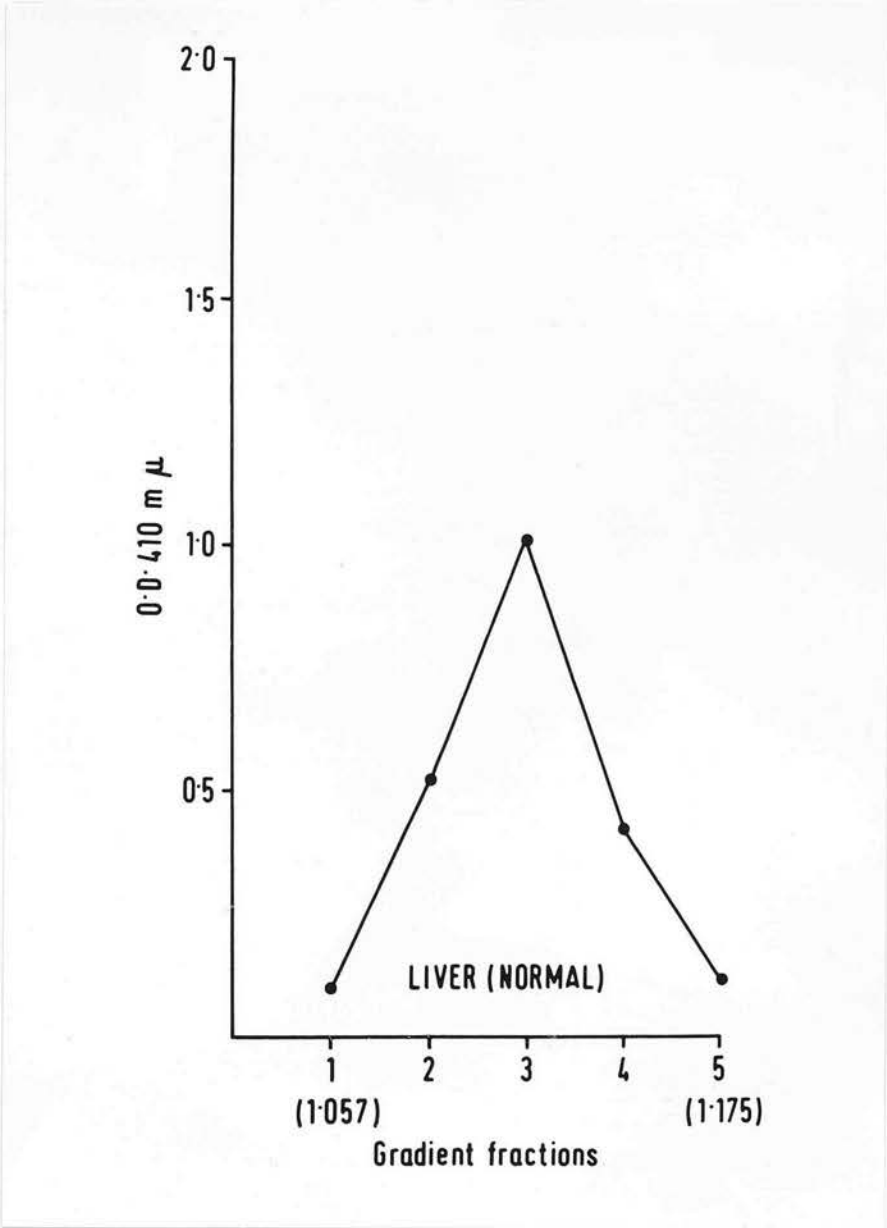


Figure 5. Acid phosphatase activity of the large granular fraction of normal liver. The peak enzyme activity lies in the region with a density of 1.125. Each point represents the mean of 6 animals.

In Figure 6, which represents the enzyme assay on density fractions of the normal, control spleen studies, the peak activity was in the density region of 1.100. The maximum quantity of detectable enzyme in this peak was 1.5 (O.D. 410 mu).

Although the spleen demonstrates higher acid phosphatase activity than does the liver, it should be pointed out that since the results do not represent the entire organ, in the case of the liver, the activity of the liver must be considered to be much higher than that of the spleen.

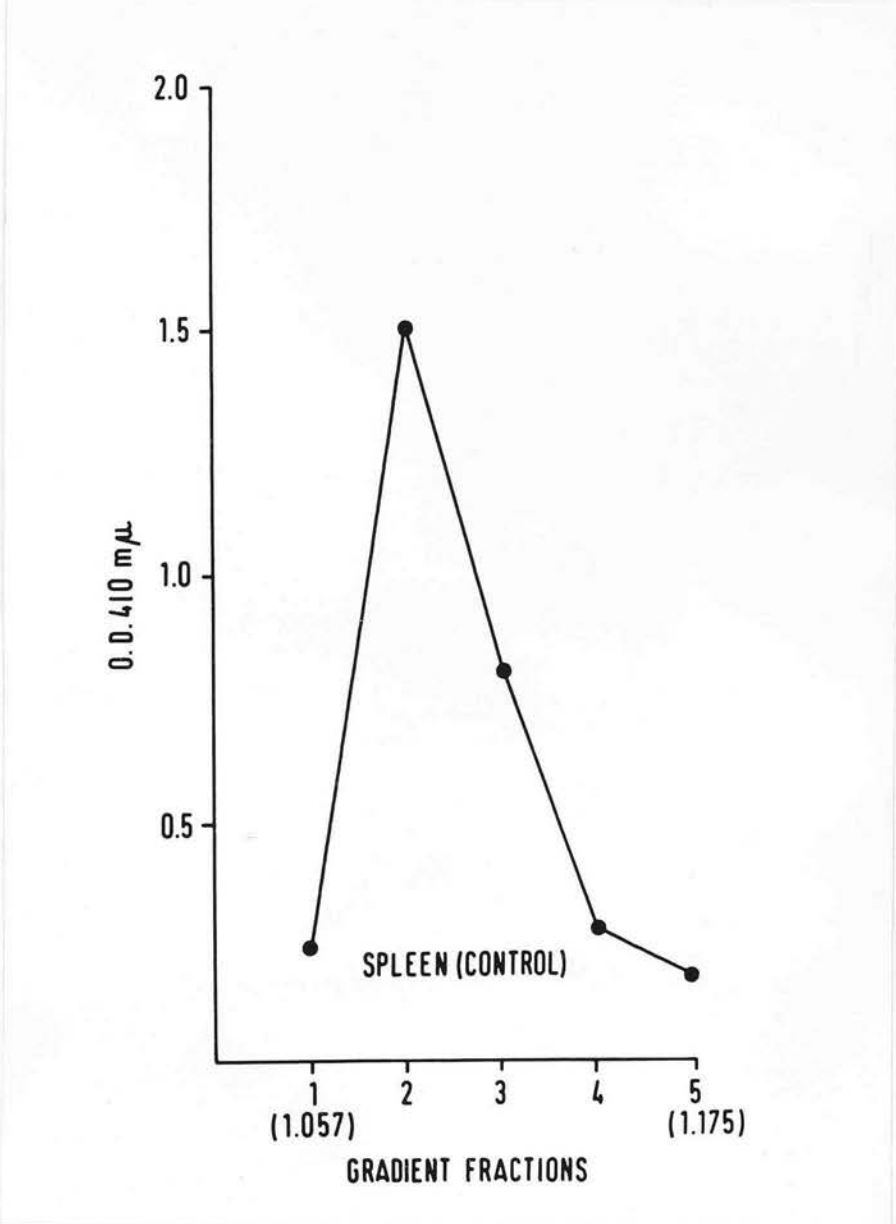


Figure 6. Acid phosphatase activity of the LG fraction of normal spleen. The peak enzyme activity lies in the density region of 1.100. Each point represents the mean of 6 animals.

Section (5) The effect of freezing and thawing on the large granular fraction of normal, non-stimulated animals

A prime characteristic of the lysosome concept is the structure-linked latency of the hydrolytic enzymes within the organelles. This may be defined as the permeability of the lysosomal membrane to the enzyme substrate. The freezing and thawing of this organelle caused the enzymes to be released and easier access to the substrate was made possible.

As previously described, the large granular fraction of tissue homogenates was prepared. Splenic homogenates were submitted to two cycles of freezing and thawing prior to their being layered onto the gradient of Urographin. The disruption procedure having been completed, the gradient with its freeze thawed preparation was allowed to centrifuge at 37,000 R.P.M. for 16 hours. The various fractions were collected and the aliquots were assayed for acid phosphatase.

The results, seen in Figure 7, show that in a density range of 1.125 to 1.250, the normal, non-stimulated animal reveals a similar activity pattern to that seen in Figure 6. The peak activity occurs in the 1.125 region and the maximum reading obtained was 1.45 (O.D. 410 mu). If the preparation was allowed to freeze and thaw, twice, prior to the density gradient centrifugation procedure, there followed a release of enzyme activity which had a maximum value of 2.0 at an O.D. 410 mu. This release of enzyme activity confirms the presence of intact lysosomes and upon disruption of the lysosomal membrane, there is a release of the enzyme activity into the surrounding medium.

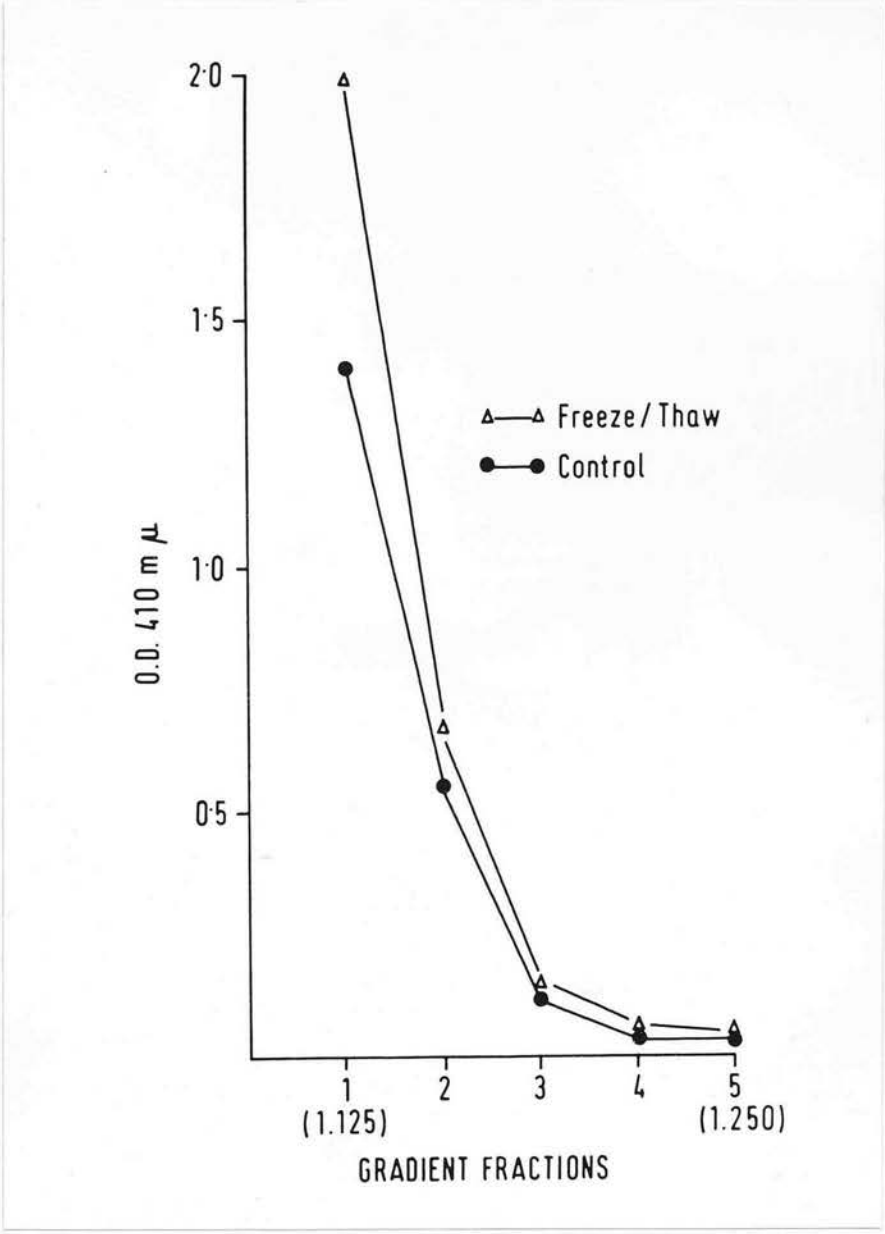


Figure 7. Acid phosphatase activity of both intact and disrupted lysosomes. The density region is 1.125 to 1.250.

Section (6) The effect of heat-denatured BSA (H.BSA) on the enzyme activity of the lysosomal population 48 hours after antigenic stimulation

In section 4 the normal, control enzyme profile of the liver and spleen had been established and the effect of antigen on this profile was examined. The two organs examined were the liver and spleen of CBA mice. The liver was studied because it represents, from the immunological point of view, a phagocytic mass of tissue which is not as actively involved in the immune response whereas the spleen, on the other hand, participates in the immune response and also has phagocytic powers.

An injection of 0.05 mg heat-denatured BSA was administered, intraperitoneally, to groups of animals. Forty-eight hours after antigenic stimulation the animals were sacrificed, their liver and spleen removed and placed into 0.29M sucrose. The same homogenization procedure was carried out as described in the Materials and Methods chapter, on the preparation of the large granular fraction. The LG fraction was then layered onto the Urographin gradient and after 16 hours, the fractions collected were assayed for acid phosphatase.

The results, shown in Figures 8 and 9, represent the liver and spleen, repsectively, 48 hours after antigenic stimulation with heat-denatured BSA.

Figure 8, which represents the enzyme activity of the liver, reveals no difference in the enzyme profile obtained in the normal animals (see Figure 5) and that obtained in animals treated with H.BSA. Both of these Figures have a maximum enzyme activity of 1.0 at an O.D. 410 mu and this activity was found to be localized in the same density region, that being 1.125.

In the case of the spleen, there is a significant difference between the H.BSA treated animals and the non-stimulated control. The control animal which gave a maximum enzyme reading in the 2nd fraction, had an

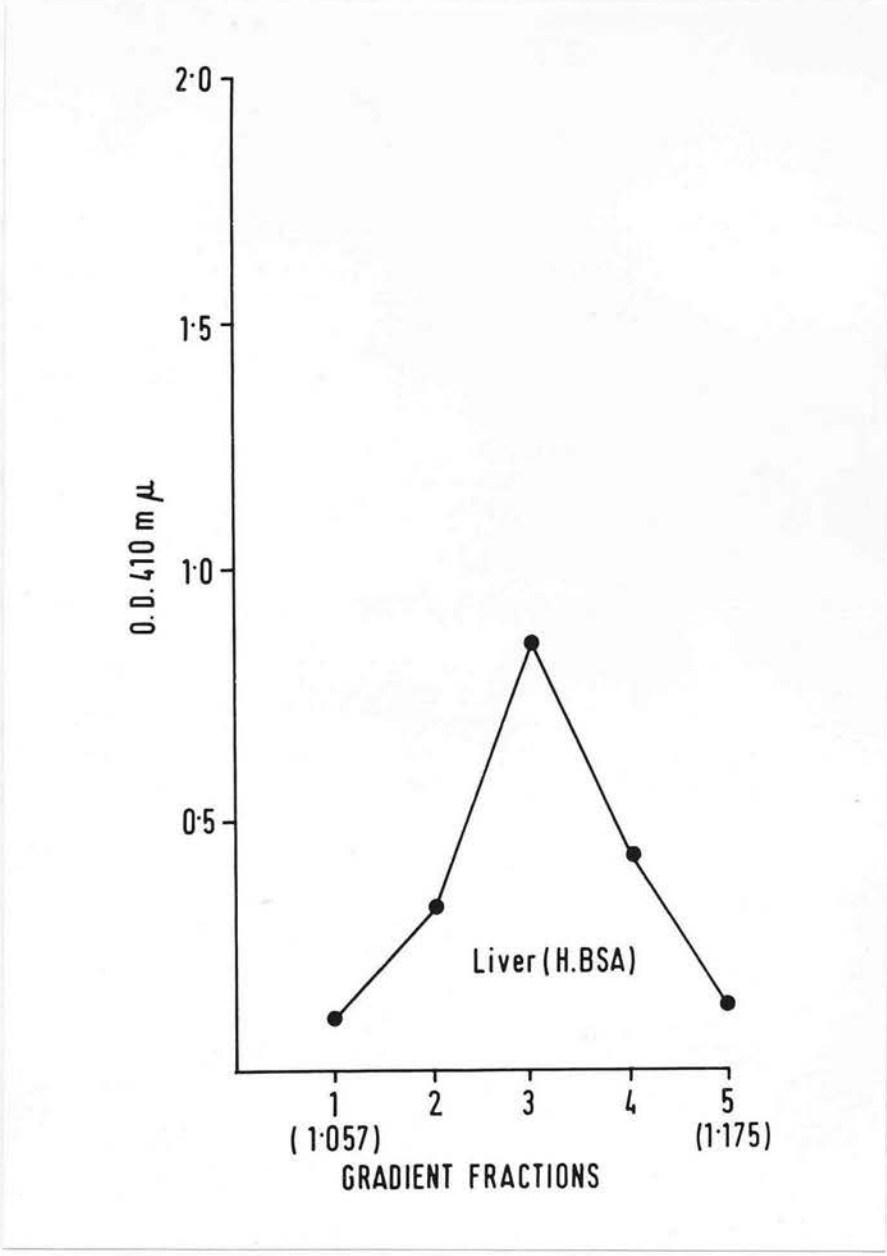


Figure 8. Acid phosphatase activity of the intact large granular fraction of the liver, 48 hours after antigenic stimulation with H.BSA. Each point on the graph represents the mean of 6 animals.

enzyme peak activity of 1.5 at O.D. 410 mu. The H.BSA treated animal, while following the same enzyme profile as exhibited in the control, showed a depression in the amount of detectable acid phosphatase present. A reading of 0.8, in the case of the H.BSA treated animal and a reading of 1.5 in the control animals reveals the difference between these two groups of animals. In each case, each group of animals were subjected to the same procedures except that one group received 0.05 mg H.BSA.

The results obtained with normal (see figure 6) and H.BSA (see figure 9) are similar to those obtained in previous experiments (see Table III) indicating the reproducibility of this technique.

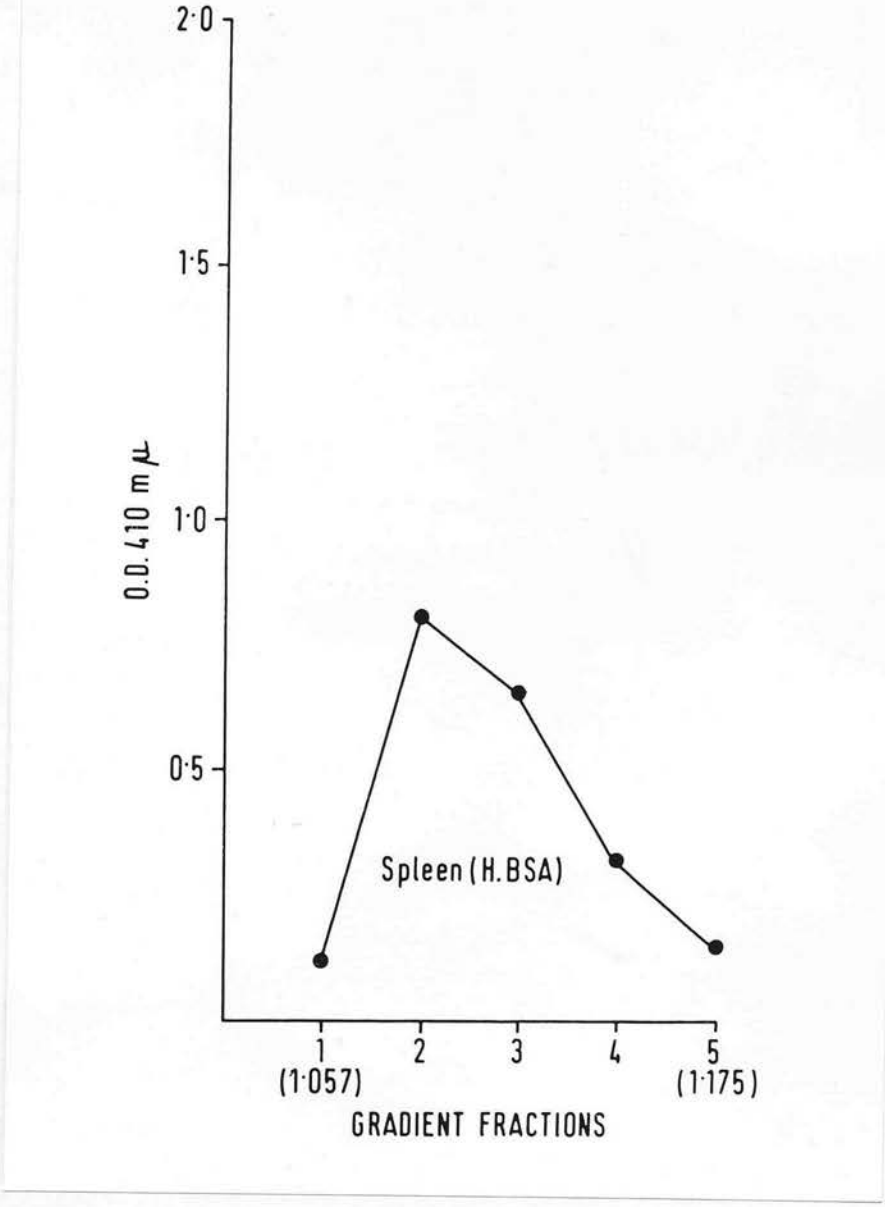


Figure 9. Acid phosphatase activity of the intact large granular fraction of the spleen, 48 hours after antigenic stimulation with H.BSA. Each point represents the mean of 6 animals.

Section (7) The effect of aggregate-free BSA(S.BSA) on the enzyme activity of the lysosomal population 48 hours after antigenic stimulation

The effect of C.BSA, upon injection of the antigen into CBA mice, results in the induction of tolerance. Examination of the lysosomal enzyme profile after stimulation of the animals with CBSA was studied.

As in the previous two experiments, groups of 3 animals were set up and each animal received an intraperitoneal injection of 0.05 mg C.BSA. Forty-eight hours after the administration of the antigen, the animals were sacrificed. The liver and spleen of each animal was removed into 0.29 M sucrose. Again the homogenization procedure, previously described, was carried out and the large granular fraction was obtained.

Gradients of Urographin, with a density range of 1.057 to 1.175, were centrifuged at 37,000 R.P.M. for 16 hours after which time the various fractions were collected. The enzyme assay on each fraction was carried out and the released p-nitrophenol was read at an O.D. of 410 mu. The effect of C.BSA, on the liver and spleen LG fraction, after density gradient centrifugation, is shown in Figures 10 and 11.

Figure 10, which represents the acid phosphatase activity of the liver, reveals an increase in the amount of acid phosphatase activity of the liver, although the density peak at which the increase occurs is the same as that seen in the normal, non-stimulated control animal (see Figure 5) as well as the H.BSA treated animal (see Figure 8).

In the case of the spleen, represented by Figure 11, there is also an increase in the detectable quantities of acid phosphatase. It should be noted that the observed increase, above the normal level, was also accompanied by a shift in the density, i.e. this shift towards the density region of 1.125 was not observed in either the normal of H.BSA

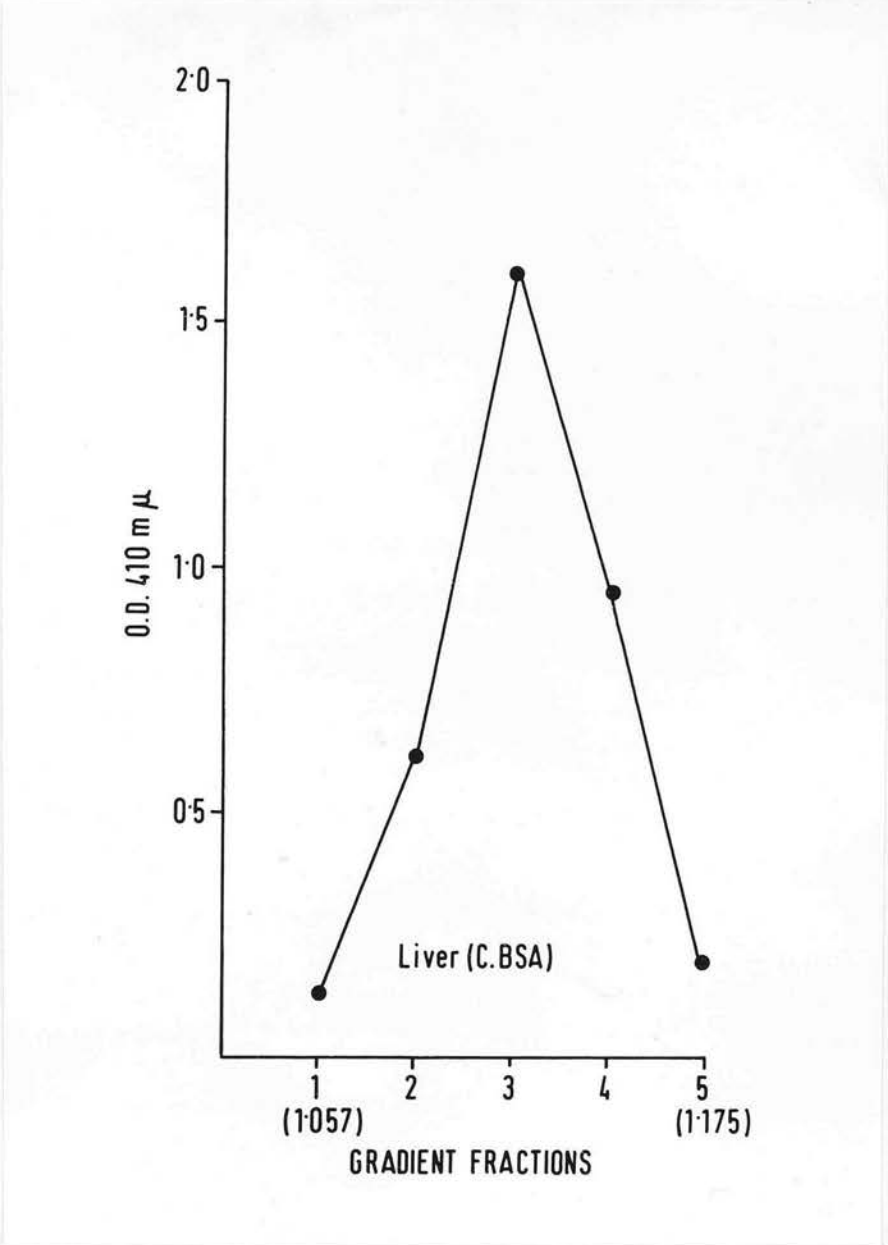


Figure 10. Acid phosphatase activity of the intact large granular fraction of the liver, 48 hours after the administration of 0.05 mg C.BSA. Each point represents the mean of 6 animals.

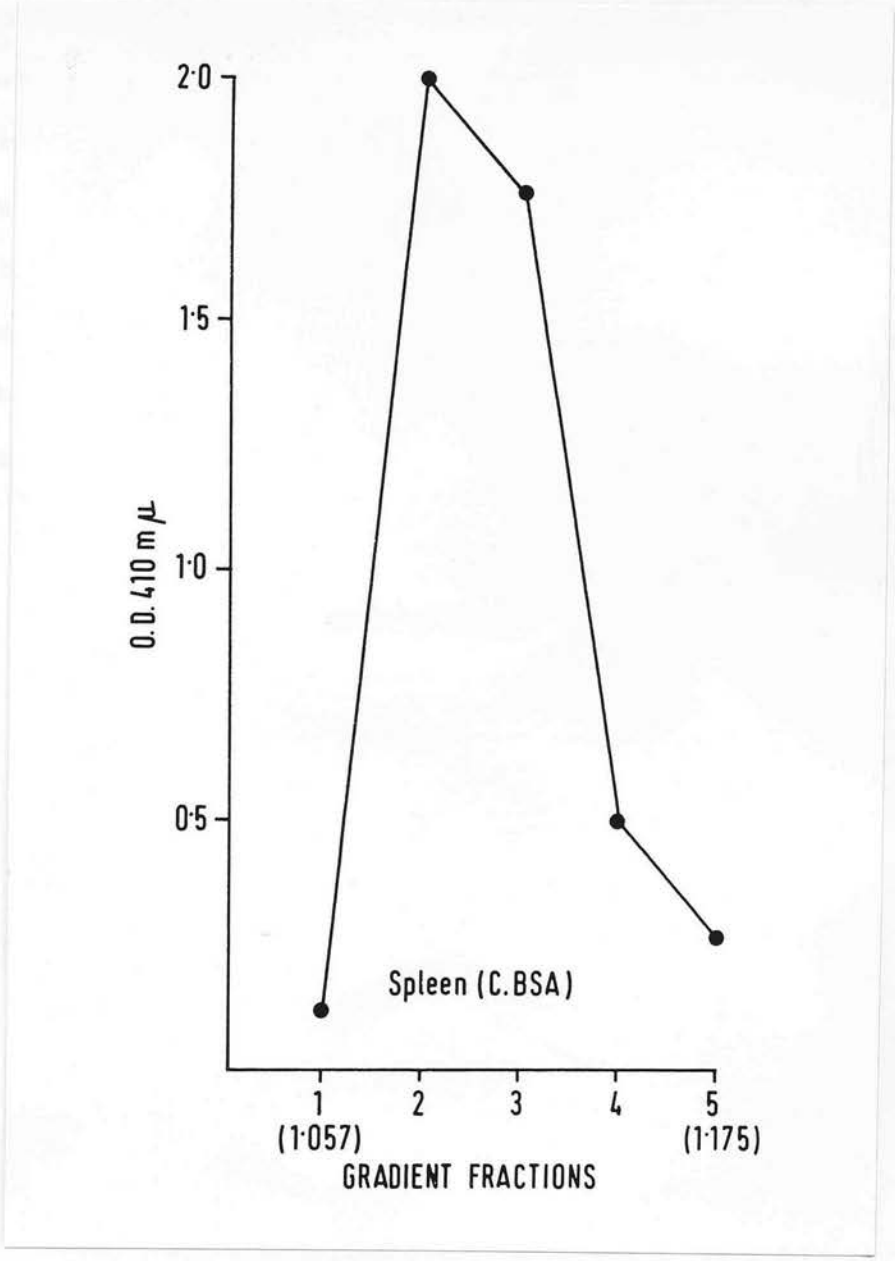


Figure 11. Acid phosphatase activity of the intact large granular fraction of the spleen after the administration of 0.05 mg. C.BSA. Each point represents the mean of 6 animals.

acid phosphatase enzyme profiles.

The difference between the H.BSA (0.8) and the C.BSA (2.0) illustrate a marked difference in the enzyme profile of these two experimental groups. In each case, the animals were given 0.05 mg of the respective antigen, the LG fraction was prepared in the same manner with each antigen studied, yet this marked difference occurred after the administration of the two antigenic forms of BSA.

The significance of these findings with respect to the stimulation of antibody production and the induction of tolerance will be discussed in the next chapter.

Section (8) Lysosomal enzyme changes over a period of time after antigenic stimulation

In the previous experiment, it has been shown that 48 hours after the administration of antigen there is an observable change in the detectable acid phosphatase content of the LG fraction, as compared to the normal, non-stimulated animal. The tolerogenic C.BSA brought about an increase in the acid phosphatase levels of the spleen whereas the administration of H.BSA led to a decrease in the enzyme level of the spleen as compared to the control animal.

In this present experiment, the enzyme changes were followed at various times after the injection of antigen. The animals were given their respective forms of BSA and at days 2,6 and 8, the large granular fractions of the spleen were assayed for the acid phosphatase levels of the organ.

Figure 12 illustrates the results obtained. After a period of 8 days, following the introduction of antigen via intraperitoneal injections, the levels of acid phosphatase were seen to return to the normal control values.

If the antigen administered were haemocyanin, which is an immunogenic material, similar to H.BSA (see Figure 4), 48 hours after antigenic stimulation the enzyme levels were depressed but had returned to the normal enzyme quantities after a period of 8 days. The haemocyanin followed the same enzyme changes as did the H.BSA. In the case of the haemocyanin, the LG fraction was employed rather than the gradient studies. Nonetheless as shown in Figure 13, the enzyme levels followed the same pattern as that seen in the H.BSA treated animals.

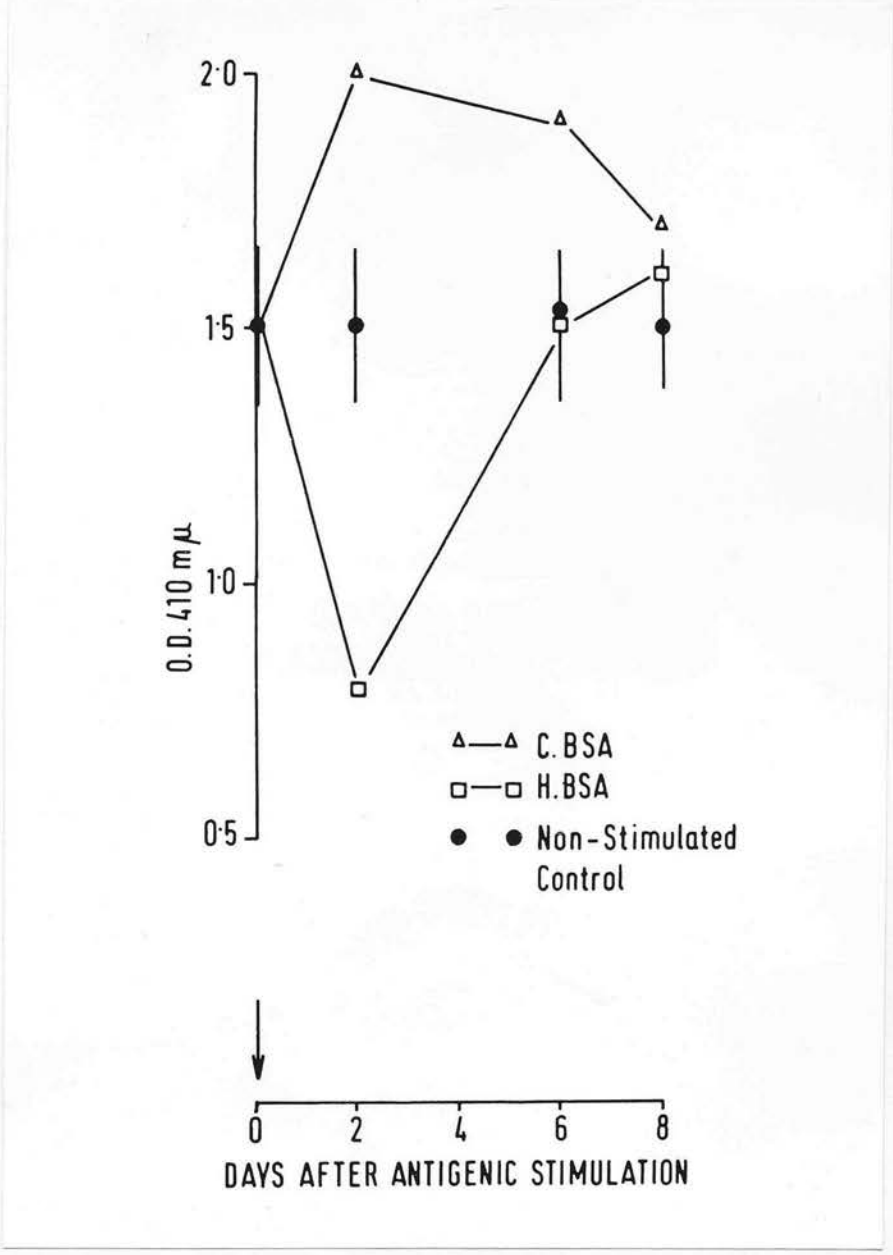


Figure 12. Acid phosphatase activity at various times after antigenic stimulation. The antigens employed were either H.BSA or C.BSA. Each point represents the mean of 6 animals.

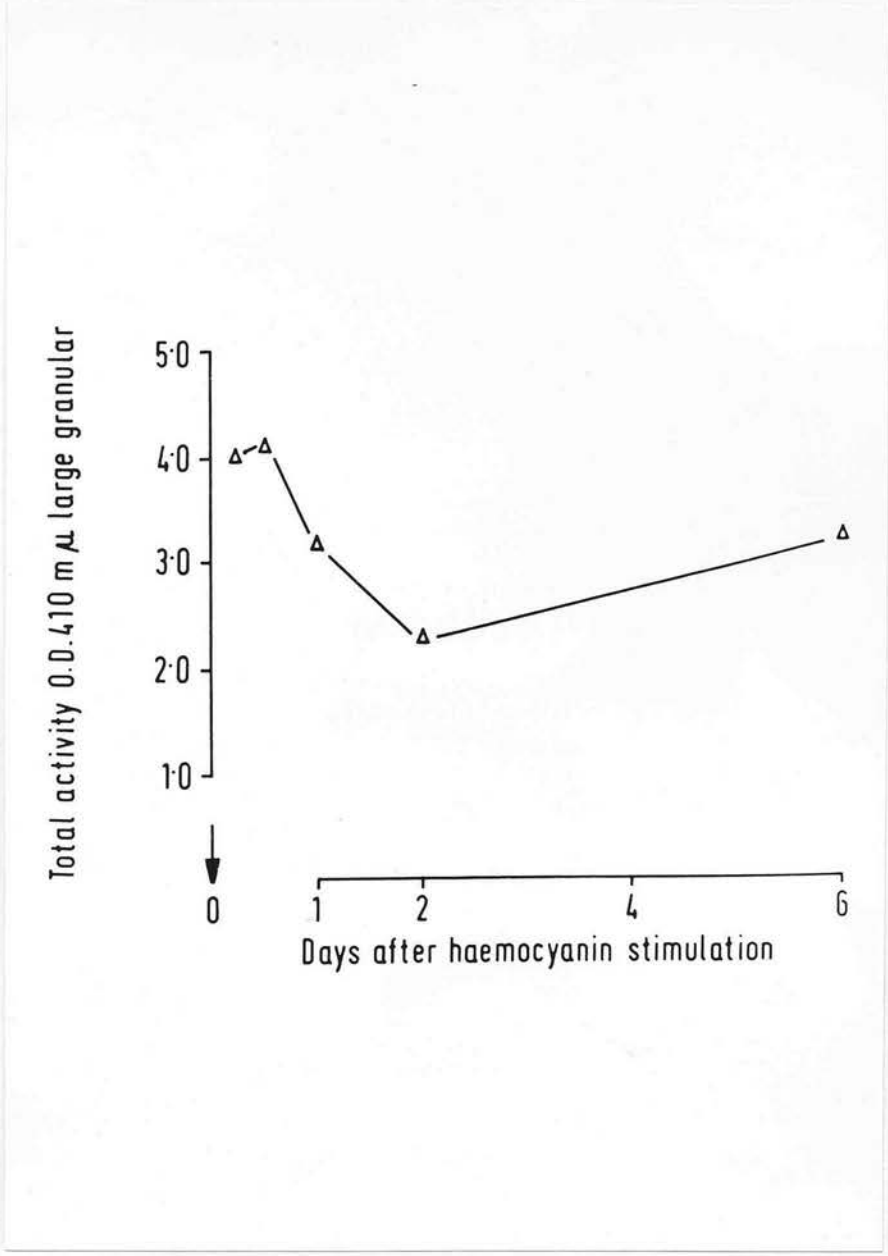


Figure 13. The acid phosphatase activity of the large granular fraction after stimulation with haemocyanin. The activity represented is the total enzyme activity of the LG fraction. Each point represents the mean of 4 animals.

Section (9) The induction of tolerance in CBA mice

The induction of tolerance has been achieved in various species employing a wide variety of antigens. Furthermore, it has been shown that the physical state of the antigen appears to be of prime importance as to whether tolerance is induced or the synthesis of antibodies occurs.

From the studies of Dresser (1962) it was shown that soluble, aggregate-free bovine gamma globulin (BGG) would induce tolerance in mice. It was the aim of this project to study the mechanisms underlying tolerance induction to BSA with respect to the involvement of the lysosomal enzymes. The soluble, aggregate-free form of the antigen was prepared as outlined in the Materials and Methods chapter.

Groups of CBA mice were given varying doses of centrifuged BSA (C.BSA) at day 0. After allowing 20 days to pass, the experimental animals were given a booster dose of 5 mg alum precipitated BSA. These antigens were prepared as previously described in the Materials and Methods chapter.

The animals were then bled on days 12 and 20, respectively, and the sera were analysed for the presence of anti-BSA antibody. Using the Farr technique, the Antigen Binding Capacity (ABC) of each of the sera was calculated. The results of this experiment are found in Table IV.

With the induction of tolerance having been established with a single, intraperitoneal injection of 0.05 mg C.BSA, the same quantity of antigen was given to a further group of animals, however, the antigen was given in a heat-denatured form.

If the physical state of the antigen is an important prerequiste for the induction of tolerance to BSA, perhaps the heat-denatured form of the antigen, which is no longer soluble, aggregate-free material

TABLE IV

The Induction of Tolerance to Centrifuged BSA (C.BSA) in CBA Mice

	Dose (mg)	Ag Conc. .1γ Day 12	.1γ 20	.01γ 12	.01γ 20
(a)	10 + challenge	3.60(4)	1.09(4)	0.69(5)	0.98(4)
(b)	5 + challenge	5.63(4)	4.14(5)	0.49(6)	1.47(5)
(c)	2 + challenge	0.30(5)	2.26(5)	0.20(5)	0.45(5)
(d)	.05 + challenge	0.00	0.06(6)*	0.00	0.04(7)**
(e)	Saline + challenge	0.44(6)	5.26(4)	0.08(6)	1.30(4)

* Significant $p<0.01$

** Represents the number of CBA mice/group.

Note : The increasing quantities of antigen administered have been shown to lead to an increase in the antibody avidity (0.01γN/0.1γN x 100). This suggests that the higher doses of material are priming the animal thus inducing a secondary response after the administration of the boosting dose (the avidity ranges from 90% to 25%).
(a = 90%; b = 36%; c = 20%; d = 60%; e = 25%).

would supply a clue to the mechanisms involved in the induction of tolerance.

Each animal was given a single intraperitoneal injection of 0.05 mg heat-denatured BSA (H.BSA). On day 20 the animals were given a challenging dose of 2 mg BSA. As shown in Table IV this quantity of antigen indicated its ability to prime the animal upon later challenging. The animals were bled at day 8 and 18 respectively, and again the sera were analysed by means of the Farr Technique in order to determine the quantity of anti-BSA antibody present. Table V illustrates the results obtained.

Although only 50% of the animals gave an ABC value at day 18, nonetheless, the H.BSA appears to be more immunogenic that was the same quantity of C.BSA.

It should be noted that the doses and schedule of antigenic stimulation in the priming effect of 0.05 mg H.BSA have been altered. Previously (see Table IV), it had been shown that 2 mg of antigen did not initiate an antibody response. Therefore it was decided that upon injection of 0.05 mg H.BSA, the later challening dose would be 2 mg BSA. If a response occurred, the possible priming effect of 0.05 mg H.BSA could then possibly be entertained.

TABLE V

The Effect of Heat-denatured BSA on the Induction of Tolerance in CBA Mice.

	ANTIGEN BINDING CAPACITY	
	0.01 % N H.BSA	0.01 % N BSA
DAY 8	-	ND
	0.61	ND
	-	ND
	-	1.30
	-	-
	0.99	0.54
DAY 18	-	-
	-	-
	-	-
	0.66	0.47
	0.40	0.36
	0.60	0.40

Section (10) The localization of isotope-labelled C.BSA and H.BSA in the liver and spleen of CBA mice

The C.BSA given in a dose quantity of 0.05 mg was shown to induce tolerance in all of the animals tested. The H.BSA, however, gave a secondary response in half of the animals tested and although not all the animals were primed it was still a more potent immunogen than the C.BSA. The isotope-labelling of antigen with either I^{131} or I^{125} enables the investigator to establish the presence of the antigen in the tissues studied. It is also possible to correlate the morphological structures as well as cell types which are involved in the uptake of antigen.

Labelled BSA was prepared, as outlined in the Materials and Method chapter, by the addition of 4 mg BSA to 4 mc I^{125}. The preparation was diluted so that the final concentration of antigen was 0.05 mg./ml. The centrifuged as well as heat-denatured BSA was injected intraperitoneally into CBA mice. These animals were removed from the departmental animal house and for the duration of the experiment were isolated in the radioisotope laboratory. The animals were sacrificed 28 hours after antigenic stimulation.

Organ counts

After removal of the tissues to be examined the various organs were weighed, counted and the amount of radioactivity per mg tissue was calculated. The results, shown in Table VI, reveal that although the centrifuged material was capable of tolerance induction and the heat-denatured antigen was more immunogenic than the former antigen, there was no significant difference in the organ counts between either form of BSA. The quantity of material, irrespective of its physical form, was present in the same amounts in the organs examined. The

TABLE VI

Localization of I^{125} - labelled BSA in the Liver and Spleen of CBA Mice

Counts per mg tissue/second

Antigen	Liver	Spleen
C.BSA	7.52(*)	8.85
H.BSA	6.45	7.69

Note : The tissues were counted for 100 seconds. The background activity was less than 1 count/second. These tissues were subsequently used in the autoradiography experiment (see page 110).

enzyme changes observed must therefore be due to the effect of the physical state of the antigen on the lysosomes rather than the amount of material reaching the organ. This amount has been shown to be the same.

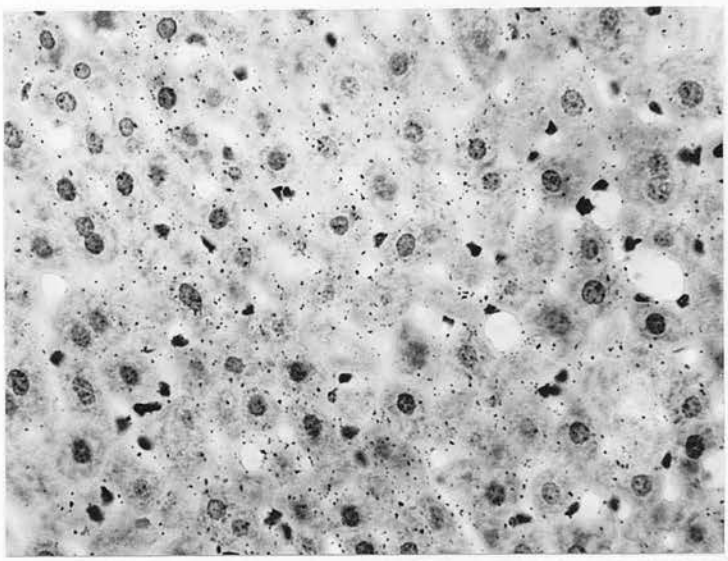
Autoradiographic studies on I^{125}-labelled antigens

Autoradiographic studies of isotope-labelled antigens enable the investigator to examine the distribution of antigen in relation to the cell type which controls the distribution pattern.

The experimental animals were injected with the two forms of BSA, which had been labelled with I^{125} (1mg/5mc) the heat-denatured antigen as well as the aggregate-free material, and 28 hours after the administration of the material the animals were sacrificed. The tissues were removed and allowed to fix in formal saline for 16 hours. For practical purposes this was usually allowed to occur overnight. The fixed tissue was then processed according to the single embedding chart illustrated in the Materials and Methods section. The tissues were embedded in paraffin, were sectioned and the slides were then deparaffinized to water. The nuclear emulsion was applied and the slides were placed into a light-proof container and kept at 4° for 2 to 3 weeks. The exposed slides were developed and stained and the results may be seen in plates 1 to 4.

The localization of C.BSA and H.BSA in the spleen and liver reveal that although both antigenic forms are localized within the tingable body macrophages (dendritic macrophages), the H.BSA appears to localized to a greater extent.

Plates 1 and 2 illustrate the lack of localization in the liver. The C.BSA (plate 1) shows endothelial cells with autoradiographic



x 450

Plate 1. Autoradiograph of I^{125}-labelled C.BSA in the liver of CBA mice.

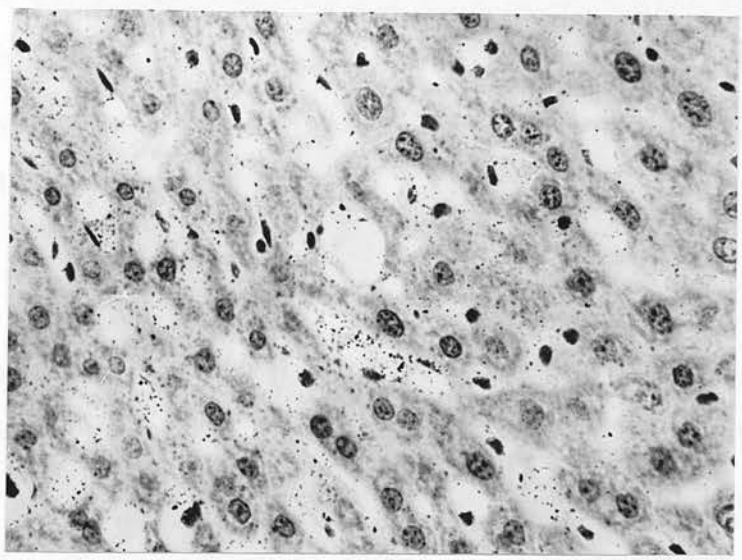


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Plate 2. Autoradiograph of I^{125}-labelled H.BSA in the liver of CBA mice.

grains localized in the cytoplasm, also the parenchymal cells reveal some cytoplasmic localization. The Kupffer cells show little uptake of the antigen. The H.BSA (plate 2) follows the same uptake pattern as described in the case above with C.BSA being the antigen.

Plates 3 and 4 represent the localization of the C.BSA and H.BSA in the spleen. Plate 3 illustrates two different stains, plate 3a which was a haematoxylin and eosin stain, while plate 3b represents the same organ sections which have been stained with methyl green pyronin.

The C.BSA (plate 3) illustrates a diffuse grain pattern. Grains may be seen over the entire red pulp with some macrophage participation in the uptake of the antigen. There is also heavy localization of grains in the circulatory vessels of the spleen.

In the case of the H.BSA (plate 4) there is heavy uptake of the antigen in the red pulp. The macrophages plus the marginal zones of the germinal centres reveal heavy antigen localization. The antigen may be seen within the dendritic processes of the tingible body macrophages being channeled through the cytoplasm to the germinal centres. There is also pyroninophilic blast cell transformation in the red pulp, occurring in the vicinity of the red pulp macrophages.

The localization of the C.BSA and H.BSA may be summarized as follow The C.BSA localizes within the macrophages of the splenic red pulp but is rather diffusely distributed over the entire organ. On the other hand, the H.BSA is localized more specifically within the red pulp macrophages.

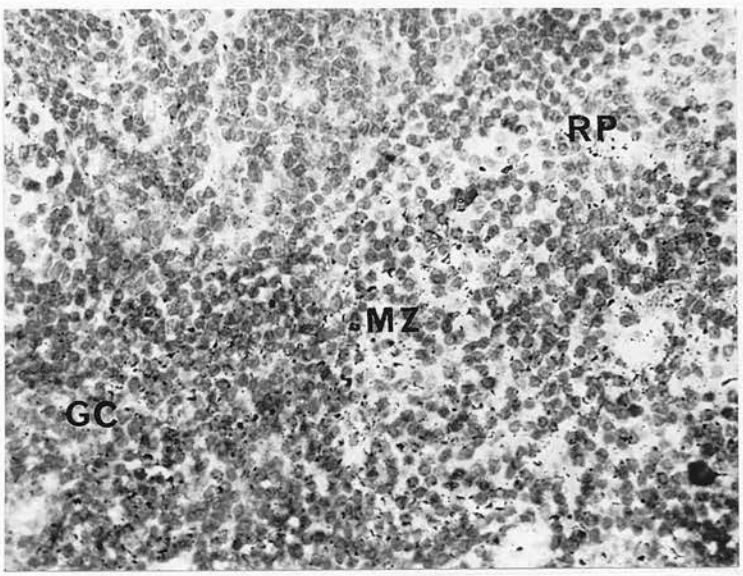

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Plate 3a. Autoradiograph of 1^{125}-labelled C.BSA in the spleen of CBA mice. The germinal centers (GC), marginal zone (MZ) and red pulp (RP) all illustrate a diffuse grain pattern with limited macrophage uptake. (Haematoxylin-eosin).

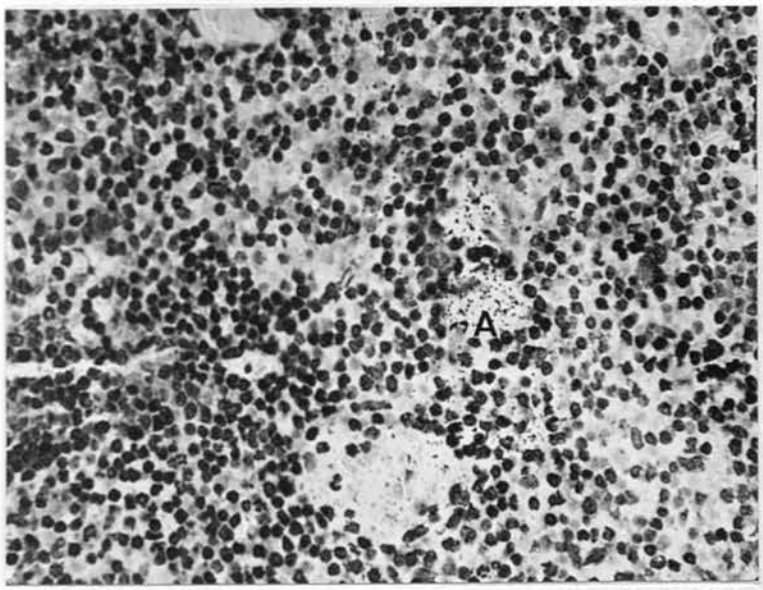

Plate 3b. Autoradiograph of 1^{125}-labelled C.BSA in the spleen of CBA mice. There is limited macrophage uptake of the antigen with the circulation revealing the presence of some label in the arterioles (A). There is a general diffuse localization pattern. (Methyl Green Pyronin).

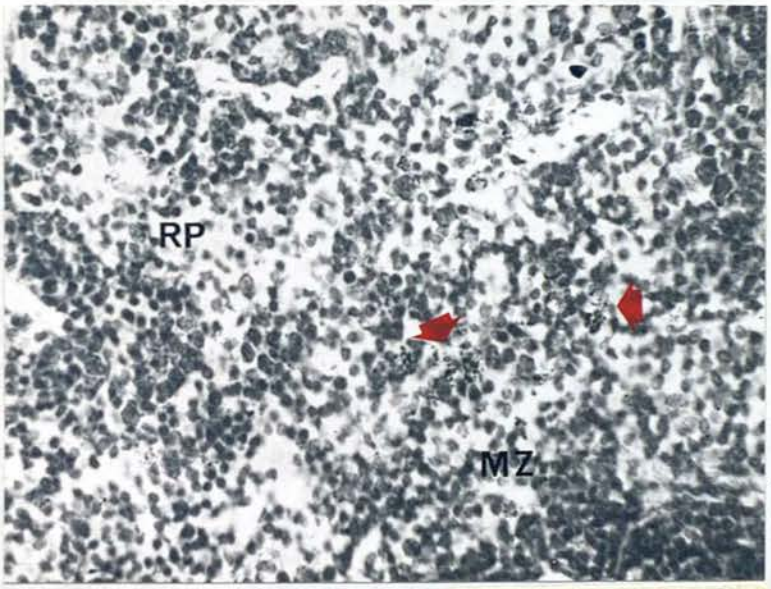

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Plate 4a. Autoradiograph of I^{125}-labelled H.BSA in the spleen of CBA mice. The arrows denote heavy macrophage uptake of the antigen in the marginal zones of the germinal centers. The red pulp also illustrates the localization of the heat-aggregated antigen. (Haematoxylin-eosin).

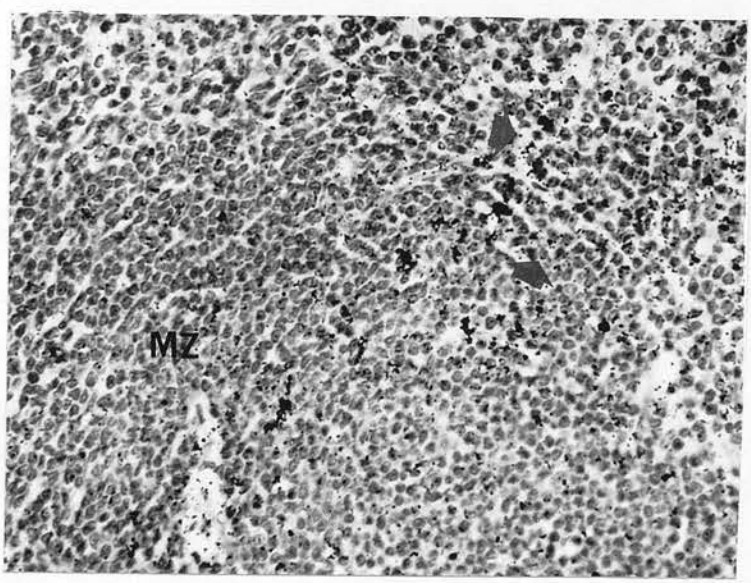

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Plate 4b. Autoradiograph of 1^{125}-labelled H.BSA in the spleen of CBA mice. As indicated by the arrows, there is heavy grain localization in the macrophages of the red pulp and marginal zones of the germinal centers. (Methyl Green Pyronin).

Section (11) The column separation of splenic lymphocytes after antigenic stimulation

The technique of glass bead column separation of splenic lymphocytes enables not only the study of the lymphocytes of the spleen but also enables the study of two forms of lymphocytes to take place: the normal, non-sensitized lymphocyte as well as the activated, antibody-producing cell.

The observed changes in the acid phosphatase levels after the injection of the two forms of BSA led to the question as to which of the many cell types in the spleen were responsible for this effect.

To ensure that a separation of antibody-producing cells from thos not involved in the synthesis of globulins, i.e. resting cells, did occur, animals were initially primed with sheep erythrocytes, 2 x 10^8 cells per ml. The animals were sacrificed 10 days after the intraperitoneal injection of the antigen and the spleens were removed and placed in 10.0 ml. Hank's buffer in which the Ca^{++} and Mg^{++} ions had been omitted. The separation of the spleen cells on the glass bead column was followed as described in the Materials and Methods section. The cells were collected in aliquots of 65.0 ml. per fraction. After concentration of the cells by means of centrifugation the cell counts were adjusted so as to contain 2 x 10^6 cells per ml. To each sample of spleen cells, in a volume of 0.5 ml., an equal volume of sheep erythrocytes, 2 x 10^7 cells per ml., were added. The mixture was allowed to incubate at 4° C overnight. The following morning the number of rosette forming cells were counted and the percentage of rosette forming cells was calculated. The results, as seen in Figure 14, reveal that through this technique of column separation of splenic lymphocytes there is a separation of those cells which

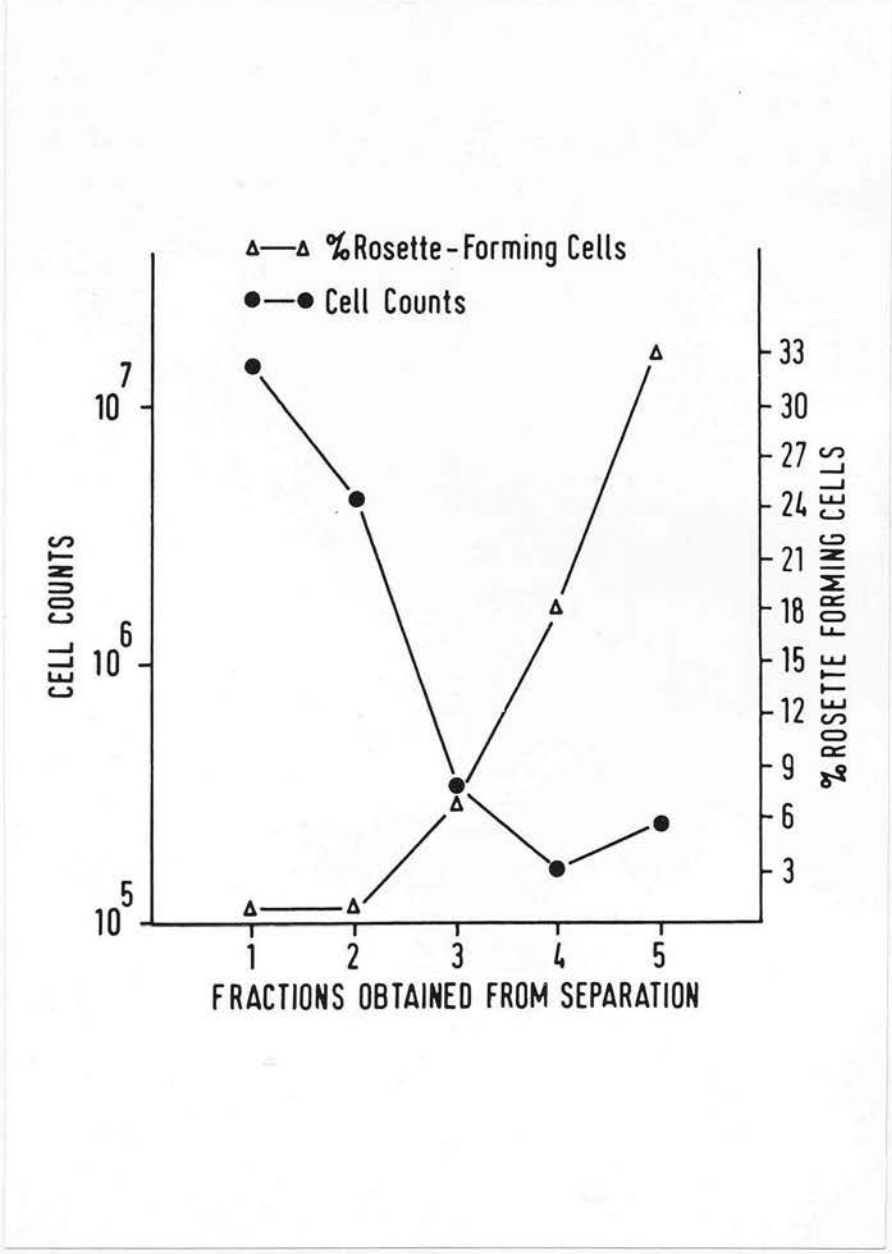

Figure 14. Column separation of splenic lymphocytes after stimulation with 2 x 10^8 sheep erythrocytes. The separation of the lymphoctyes reveals a division of normal and sensitized cells. The sensitized cells are able to form rosettes whereas the normalcells are not. Each point represents the mean of three animals.

engaged in antibody production and those which are not involved in the synthesis of antibody and consequently do not produce rosettes when mixed with the sheep erythrocytes.

Having established the separation of normal and sensitized lymphocytes, by means of column separation, the technique was applied to the separation of splenic cells after treatment with either H.BSA or C.BSA. The dose quantity tested in each case was 0.05 mg per ml. Forty-eight hours after the administration of antigen the spleens were homogenized as previously outlined and the separation of the spleen cells followed.

The results of the column separation study may be seen in Figure 15. In each case, irrespective of the antigen employed, a rise in the fifth fraction cell count occurred. This is due to the fact that all the animals tested, including the controls, were responding to the various environmental stimuli which are constantly present, and the administration of the antigen, in the case of the experimental animals, includes these responding cells.

However, the cells collected in the 5th fraction were subjected to acid phosphatase analysis and after sonication (amplitude of 8 microns, peak to peak) of 10^6 spleen cells, the total acid phosphatase content was determined on each sample of cells. The results of this assay are shown in Table VII.

TABLE VII

Acid phosphatase activity of 10^6 spleen cells after column separation of the spleen homogenates

	O.D.410 mu
control	0.15
H.BSA	0.57
C.BSA	0.58

The readings obtained for the 5th fraction cells reveals that

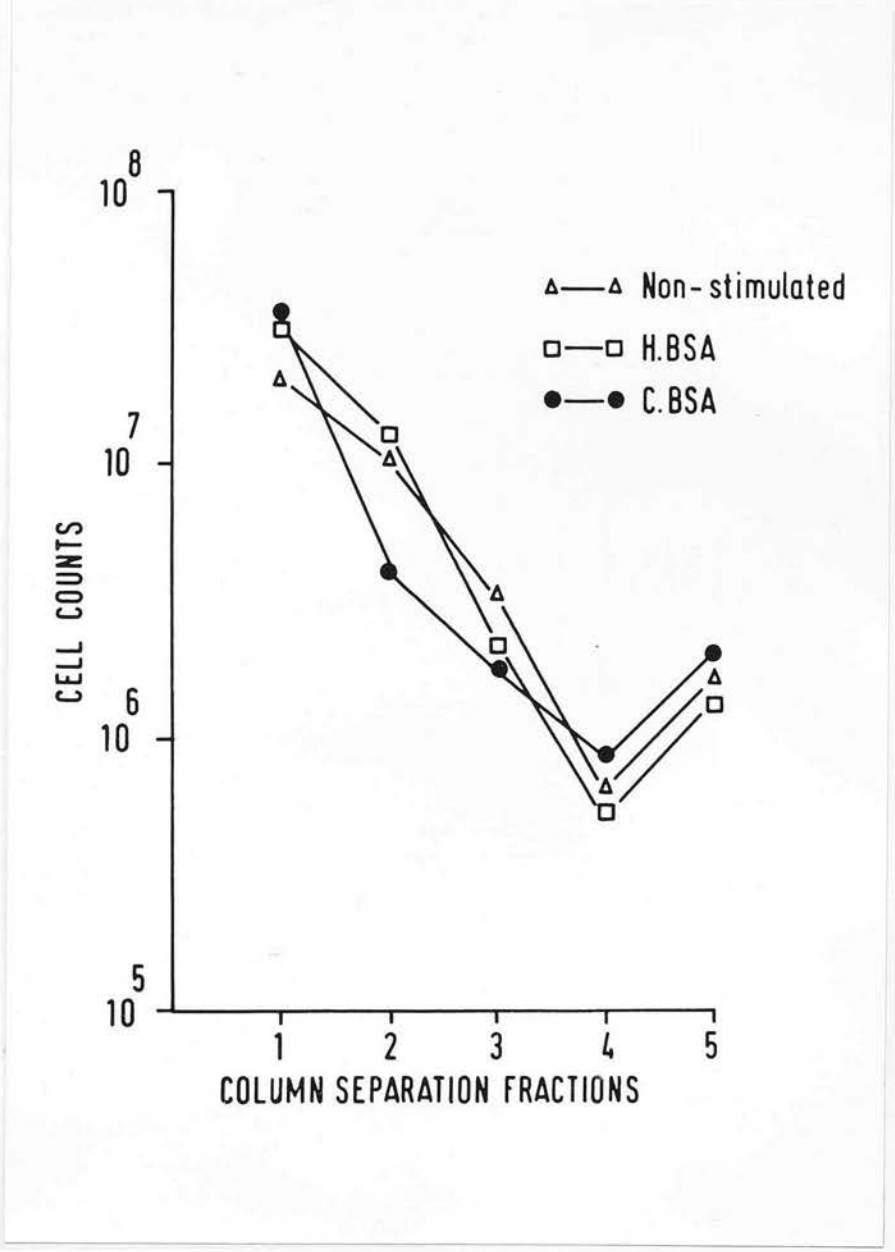

Figure 15. Column separation of splenic lymphocytes after antigenic stimulation. Three separations illustrated represent treatment with saline, H.BSA and C.BSA. Each point represents the mean of three animals.

both forms of the antigen cause an increase in the amount of acid phosphatase present, as compared to the normal, non-stimulated animals. These results are similar to those demonstrated in the first section of the experimental results in which 10^6 spleen cells were assayed for their acid phosphatase content. In each case, the experimental animals all showed higher acid phosphatase levels than did the control animals.

The possibility exists that the splenic macrophages contaminating the suspension add to the acid phosphatase values which have been obtained. Macrophages have, as one of their physiological characteristics, the attribute of being "sticky" cells. Leucopac, which in essence is cotton wool, was packed into a column and the cell suspensions were passed through the column, prior to the actual separation procedure, to ensure the removal of the macrophages.

Passage of the spleen cell suspension (pool of three organs) through the column of leucopac resulted in a loss of a factor of 10^1 cells. To check the removal of the macrophage cells, the animals were given an intraperitoneal injection of colloidal carbon (50 mg), two hours prior to the removal of their spleens. Following separation of the spleen cells, the preparations were observed microscopically for the presence of cells containing carbon in their cytoplasm. Cells which have passed through the column of leucopac had less carbon in their cytoplasm (2% to 4% of the total cell number) than did the cells which were collected normally (10% to 15% of the total cell number). If the cells from the 5th fraction were analysed for their enzyme content, after the leucopac treatment, the results illustrate that the cells from the H.BSA treated animals (three pooled spleen cell suspensions) gave a reading of 0.12 O.D. 410mu, whereas the cells which had not passed through leucopac columns gave readings of 0.19 O.D. 410mu.

Thus although macrophages had been removed, the "purified" lymphocyte preparations nonetheless gave detectable acid phosphatase readings.

The results obtained in this experiment represent the enzyme level of disrupted lysosomes. The cells examined were subjected to 10 seconds sonication (amplitude of 8 microns, peak to peak) prior to the acid phosphatase assay. It would have been desirable to study the enzyme changes on intact lysosomes, but due to the small number of cells obtained from the column it was found necessary to estimate the total enzyme activity of the disrupted lysosomes. From these results, as in the experiments with 10^6 spleen cells (see Figure 2), it has been shown that after antigenic stimulation, irrespective of the antigen employed, there follows an increase in the total enzyme activity of the disrupted cells.

Lastly, it should be noted that there were no rosette forming cells found in the fifth fraction cells when assayed with antigen coated sheep erythrocytes. This seems to imply that the enzyme assay system employed is able to detect changes in antigen stimulated cells long before antibody can be detected upon their surface.

Section (12) Histochemical studies on purified lymphocyte preparations

The column separation of splenic lymphocytes and the acid phosphatase activity of these sonicated cells led to the study of the splenic lymphocytes by the available histochemical techniques.

The observed changes, in acid phosphatase activity, of the intact large granular fraction (see Figure 4) of the spleen required the identification of cell type involved in this change. The column separation studies suggest that the lymphocyte contributes to being the cell responsible for the enzyme changes. Thus it was undertaken to examine the lymphocytes of the spleen using an acid phosphatase staining procedure. The staining procedure was outlined in the Materials and Methods section and it was found that the cells required an incubation period of 24 hours with the substrate to ensure heavy lysosomal grains.

The results (seen in plates 5, 6 and 7) reveal that whether the antigen be H.BSA or C.BSA, there followed an increase in the number of lysosomal grains per cell. These results correlate with the previous results which revealed an increase in the amount of detectable acid phosphatase after treatment with antigen.

The fact that the lymphocytes appear to contribute to the total enzyme levels of the spleen may not rule out other cells taking part in the total enzyme levels. This point is not in dispute. The author merely wishes to point out that the lymphocyte participates in these changes and that this may be of importance in relation to the mechanisms of tolerance induction.

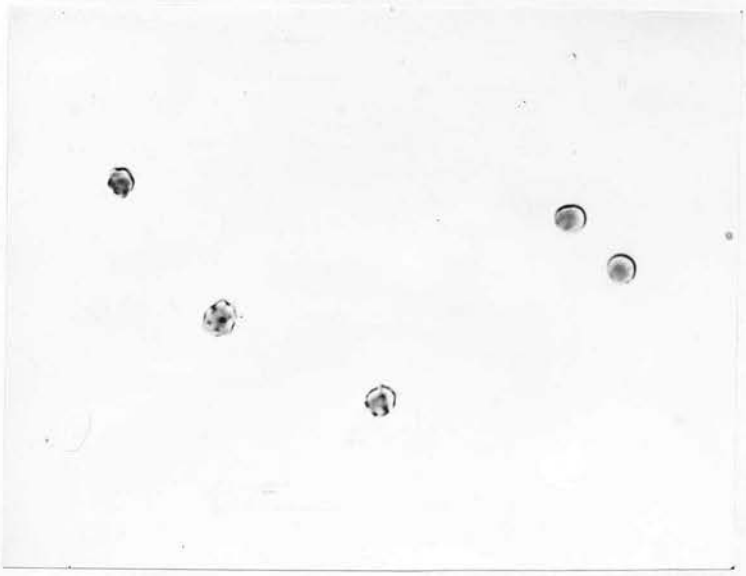

Plate 5. Histochemical staining of acid phosphatase in purified lymphocyte preparations. The lymphocytes represent normal, non-stimulated spleen cells.

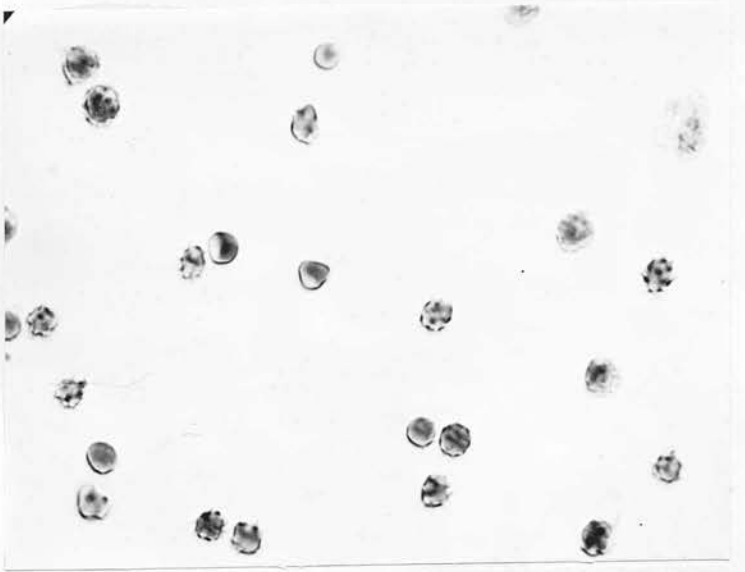

Plate 6. Histochemical staining of acid phosphatase, after treatment with H.BSA.

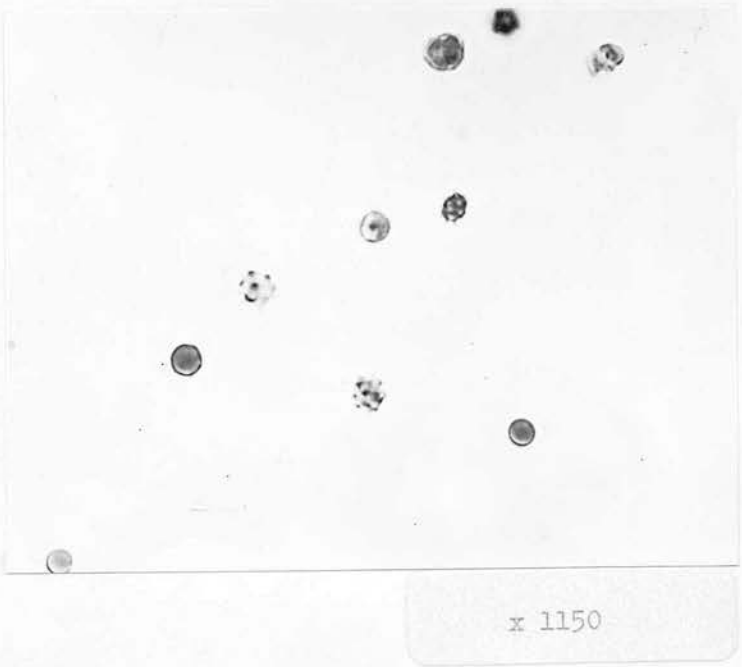

Plate 7. Histochemical staining of acid phosphatase on purified lymphocyte preparations, after antigenic treatment with C.BSA.

DISCUSSION OF EXPERIMENTAL RESULTS

Summary Chart of Experimental results on Enzyme studies

Cell preparation assayed	Acid phosphatase enzyme levels after antigenic stimulation (as compared to non-stimulated controls):	
	Immunogen	Tolerogen
10^6 disrupted spleen cells	↑	↑
Disrupted L.G. fraction	↑	↑
Intact L.G.fraction	↓	↑
Fractions obtained by Gradient separation of the intact L.G. fraction	↓	↑
Histochemical staining of purified lymphocytes	↑	↑

↑ = increase in enzyme activity

↓ = decrease in enzyme activity

DISCUSSION OF EXPERIMENTAL RESULTS

The primary purpose of this study was to observe any changes in the lysosomal enzyme levels after antigenic stimulation and to relate the physical state of the antigen to the accompanying enzyme changes.

Although there is a vast quantity of information in the literature on the role of the lysosome in many biological processes, little information exists on the lymphocyte-lysosome relationship with respect to the production of antibodies and the induction of tolerance. Many of the ideas put forth, must of necessity, be of speculative nature but from the evidence presented, the possibility exists whereby lysosomes may play an important role in the antibody-producing stage of the immune response as well as in the inductive phase of tolerance.

Evaluation of techniques

Prior to the discussion of the experimental results and the theoretical aspects of antibody formation and the induction of tolerance, the author wishes to evaluate briefly certain techniques which have been employed in this study.

Perhaps the procedure reported in this project which imposes the severest restrictions is that of the density gradient centrifugation studies of the large granular fraction on gradients of Urographin. The MSE Superspeed 40, with its SR40 rotor, is only capable of taking 3 gradients for each proposed experiment. This limiting factor only enables 3 animals to be studied at a given time. Furthermore, this factor of 3 must be properly controlled with each gradient trial for the antigen being studied. It is well known that with the passage of time, animals tend to vary with respect to their response upon receiving antigenic stimuli. For this reason, normal control animals were

included in each set of experiments, enabling the enzyme determinations to be under constant comparison with the original studies. It is for this reason that large numbers of animals were not involved in a given experiment at any given time. The preparation of the large granular fraction involves the homogenization of the organ by means of a mechanical homogenizer (see Materials and Methods/Preparation of the LG fraction). The LG fraction, depending on the time and degree of homogenization, will vary in the amounts of detectable acid phosphatase. This is due to the release of additional lysosomes from the tissue in question. Nonetheless, since the homogenization was kept constant, the results obtained and reported in this project through experimentation are comparable, for it is the qualitative differences, rather than quantity of enzyme detected, which has been shown to be the important factor. The separation of the large granular fraction, described by Williams and Ada (1967), employed Urographin as the solute for the isopycnic centrifugation of the spleen homogenate. Separation of the various subcellular organelles was achieved on Urographin gradients and the solute did not affect the activity of the enzymes studied (Williams and Ada, 1967). The only objection to the use of Urographin, as a solute, rests in the finding that at an optical density of 280 mu there occurred interference which would not allow protein determinations to be performed.

The technique of column separation of splenic lymphocytes has been shown to separate antibody-forming cells from cells which are not actively involved in the synthesis of antibodies (Plotz and Talal, 1967). During the experimentation carried out in this project, it has been shown that mouse spleen cells sensitized to sheep erythrocytes were capable of rosette-formation if the antigen were mixed with the

sensitized cells, but if the normal or 1st fraction cells were mixed with the sheep red blood cells, no rosette forming clusters were observed. This technique, while separating spleen cell suspensions does not necessarily separate lymphocytes from macrophages. The presence of EDTA in the final buffer will allow some of the macrophages to leave the glass bead surface thus entering the final cell fraction. For this reason, columns of leucopac were employed to remove the macrophages from the preparation prior to separation on the column of glass beads. There followed a reduction in cell number (10^1) yet the final fraction of cells, presumably lymphocytes, were still able to give detectable acid phosphatase readings. Injections of 50mg colloidal carbon, prior to the removal of the spleens enabled the various fractions collected to be examined for the presence of carbon in the cytoplasm of the cells. Accompanying the reduction in cell count was a reduction in the cells containing carbon. Non-leucopac preparations were found to contain 10% to 15% carbon-containing cells whereas in leucopac 5th fraction cells, only 2% to 4% of the cells contained carbon. Thus it was shown that the separation of spleen cell suspensions on glass bead columns did, in fact, separate cells which were sensitized from cells not actively engaged in the synthesis of antibody.

Autoradiography of spleen and liver has shown that upon injection of I^{125}-labelled BSA there follows certain localization patterns in the respective organs. In order to ascertain the degree of localization, serial sections of the organ in question were applied to a single microscope slide. After the application of the nuclear emulsion, exposure for several weeks and subsequent developing and staining of the sections; each slide was examined for the presence of autoradiographic grains in similar areas of the serial tissue sections (for example, autoradiographic grains in the marginal zone of the malpighian follicles in one section were observed in a similar area in an adjacent section).

The employment of serial section examination, in order to detect antigen localization, enabled the constant background labelling problem to be overcome. Similarly, the use of L.4 nuclear emulsion, which gives a very small grain size was replaced by the K.5 emulsion which gives larger grains on exposure to radioactive isotopes. The quantity of radioactive isotope required during the labelling of BSA and later in the subsequent autoradiographic studies was found to be high. In consultation with Professor R.G. White and Dr. M.A.B. de Sousa it was decided that labelling of BSA (1mg) with 5mC I^{125} gave satisfactory radiographic grains. The "hottness" of the label is the most important prerequiste in autoradiographic studies.

The use of histochemical staining techniques have been shown to reveal qualitative differences but not quantitative differences in the lysosome population of human peripheral blood lymphocytes. Hirschhorn and her co-workers (1965) were able to show a 500% increase in the number of acid phosphatase positive granules in their tissue culture preparations but upon biochemical analysis there was indeed an increase but not to the same extent as illustrated by the staining procedure. Due to the variations in incubation, 16 hours to 24 hours, various grades of staining were obtained. The optimal time of 24 hours provided the most satisfactory results. Provided biochemical studies accompany the histochemical staining procedures, the latter test as an indicative system may reveal enzymatic changes which are taking place. The test as stated previously may be employed to reveal qualitative but not quantitative differences.

Biochemical nature of the lysosome membrane

Although little is known, at present, about the biochemical nature of the lysosomal membrane from the first biochemical studies of de Duve and his associates (1955) it has been deduced that the envelope surrounding the hydrolytic enzymes was of a single-limiting unit nature. Tappel, Sawant and Shibko (1963) have further postulated that lysosomes possess a charged lipoprotein membrane. The physiological action of this membrane may occur through an excitation process controlled either by hormones and/or free radicals. If lysosomes do possess a charged membrane, then the availability of their enzymes, to a given substrate, would be dependent on the electrostatic binding of the enzymes within the lysosome structure. If this postulation is valid, perhaps the binding of antigen to the exterior cell surface changes the permeability of the cell membrane proper thus upsetting the internal free-radical concentration of the cell which would in turn bring about the labilization of the lysosome itself. The above workers have shown that stabilized lysosomes when incubated with varying concentrations of free radicals will release their enzymes depending on the radical concentration in the environment. Although speculative, this mechanism on the labilization of the lysosomal membranes could exist within the in vivo system of lysosome-cell interactions.

Acid phosphatase activity of 10^6 spleen cells after antigenic stimulation

Several workers have shown, mainly by means of the histochemical techniques available, that human peripheral blood lymphocytes, upon exposure to antigen and/or other stimuli, initiate the internal processes of the cell to increase their normal lysosomal level with respect to their number. This increase was determined by staining for acid phosphatase granules (Allison and Mallucci, 1964; Hirschhorn, et al., 1965). The observed increase in lysosomes was seen with both light microscopy as well as with electron microscopy and the histochemically stained granules by both these observations were similar, i.e. a general increase in their number. The use of antigen stimulated lymphocytes follows a similar pattern to that of the cells which were stimulated with PHA (Hirschhorn, et al., 1965). In this study, tuberculin-sensitive lymphocytes were cultured in the presence of the antigen and although only 20% of the cells underwent transformation this increase in acid phosphatase granules was considerably greater than in the controls, which normally were found to contain only 1 to 2 granules. Similar findings were reported by Allison and Mallucci (1964), in that in studying tissue cultures of human peripheral blood lymphocytes they too found that upon stimulation with PHA there was an increase in the acid phosphatase levels of the cells. These workers have shown that lysosomes of lymphocytes which normally remain circumferential in the cytoplasm of the cells become perinuclear during the transformation process. This involvement of the lysosomes has been proposed, by the above workers, to be an essential part of the transformation of the lymphocytes.

In this present investigation, the use of three antigenic forms has been employed. The sheep erythrocytes are the most complex

antigen, followed by haemocyanin and finally bovine serum albumin. The acid phosphatase activity in 10^6 spleen cells, for all the antigens studied, was above the normal, control levels. In each case the material injected gave rise to an increased enzyme level. However, it should be noted that the variation, in the enzyme activity detected within a given experimental group, was often large. This finding was due, in part, to the cell concentration and more importantly to the actual diluting of the cells. The initial cell counts were calculated by means of Neubauer haemocytometer and from the resulting cell concentrations the dilutions were calculated. Nonetheless, the increases over and above the normal are not in dispute, as seen in Table I. To ensure a greater sensitivity and accuracy, the use of spleen cell preparations was set aside and the large granular fraction of spleen homogenates was studied. The results of the spleen cell studies represent the enzyme activity of the lymphocyte as well as the macrophage but these cell types are diametrically opposed to each other with respect to the number of lysosomes present per cell. The macrophage lysosomes possess the full complement of hydrolytic enzymes whereas the lymphocytes possess only 1 or 2 lysosomes per cell and these organelles are deficient in many of the hydrolytic enzymes (Bowers and de Duve, 1967). In their study, they were able to separate these lysosomal populations and assayed the enzyme content of the respective fractions. The macrophage lysosomes contained all the presently known hydrolytic enzymes which are associated with the lysosomes of these phagocytic cells whereas the lysosomes from the 1.15 fraction, which are thought to be associated with the lymphocyte cell series, were found to contain only three of the hydrolytic enzymes; namely, cathepsin, acid phosphatase and beta-glucuronidase, the other enzymes were either lacking or in such small quantities that their presence could not be detected.

Acid phosphatase activity of the large granular fraction (LG) of spleen homogenates after antigenic stimulation

The differential centrifugation of tissue homogenates provides an excellent, as well as accurate tool with which to study the enzyme changes within a given organ. The preparation of the LG fraction employs the centrifugal forces of varying rotor speeds to separate the cellular debris from the essential organelles which will be studied. The initial separation was achieved by centrifugation of the homogenate at 8,000 R.P.M. (4.0×10^3 G) for 10 minutes, this force over this period of time will initially separate the large aggregates and general structural components of the organ from the smaller and less dense cellular debris. The second force applied to the sample is that of 16,000 (2.1×10^4 G) R.P.M. for a further 10 minutes; this force finally brought about the pellet formation which was found to contain many of the cytoplasmic constituents of the cells which populated the organ in question. It was the use of biochemical enzyme assays (de Duve, et al., 1955) which finally characterized the organelles present in the final pellet (see Introduction/Biochemical studies on lysosomes).

The stimulation of the experimental animals with either haemocyanin or one of the two forms of BSA again led to an overall increase in the amount of acid phosphatase detected as compared to the control animals (see Table II).

The analysis of the enzyme activity was determined after the lysosomes, as well as other organelles present, had been disrupted. Thus the results obtained (see figure 3) represent the total enzyme activity of the large granular fraction. Similarly, the enzyme activity of the 10^6 spleen cells (see figure 2) represents the total

activity of acid phosphatase present since the cells were subjected to sonication prior to the enzyme assay test. Both sonication as well as freezing and thawing have been shown to disrupt the lysosomal membranes releasing the enzymes held within to react with their appropriate substrate (de Duve, 1959). The nature of the lysosome with its structure-linked latency means that if the organelles are assayed for their enzyme activity and their membranes are still intact, there will be a lower enzyme value obtained rather than if the membrane were torn apart, which would allow the enzymes to come into direct contact with their substrate thus yielding a higher enzyme reading. Weissmann (1967) has reviewed the subject of lysosomal membrane labilization and stabilization and has listed all of the agents which have been shown to effect the lysosome membrane.

The implications of the total enzyme increases following stimulation with the various forms as well as various types of antigen will be discussed in the theoretical aspects of antibody production and the induction of tolerance later in this discussion.

Acid phosphatase activity of the intact large granular fraction after antigenic stimulation

The very nature of lysosomes, with respect to the structure-linked latency of their enzymes, is a most useful tool in the characterization of the large granular fraction. As already discussed, the disrupted lysosomes when assayed, revealed an overall increase in the quantities of detectable acid phosphatase as compared to the control levels. However, if the enzyme assays were carried out on intact organelles, the results obtained suggest that it is the permeability of the lysosomal membrane which is involved in the enzyme increases observed. This increase may be related to the

permeability of the membrane for the only variable in this series of experiments was the disruption procedure carried out on the LG fraction. Of the three antigens tested, two antigens caused a reduction in the detectable levels of enzyme, provided the LG fraction was intact, while the third antigen induced an increase in the enzyme levels with respect to the control levels. The first two antigens were haemocyanin and heat-denatured BSA (H.BSA). Both these antigenic materials are immunogenic, to varying degrees. The third material examined, namely the soluble, aggregate-free BSA (C.BSA) has been shown to induce tolerance after being injected intraperitoneally (see Table IV). From the data presented, one may interpret the above results as suggesting that the initiation of antibody synthesis is followed by or accompanied by a stabilization of the lysosome membrane. On the other hand, the induction of tolerance with the aggregate-free BSA results in the labilization of the lysosomal membrane. The implications of these findings will be dealt with in the theoretical aspects of antibody production and the induction of tolerance.

Density gradient centrifugation of the LG fraction after antigenic stimulation

The distribution of enzyme activity, employing an intact LG fraction, allows for the characterization of the enzyme profile as well as possibly identifying the cell types involved.

Examination of the liver enzyme changes, following the injection of antigen, reveals that although an increase in enzyme content results after the administration of the soluble BSA (C.BSA), the normal animal and that treated with the heat-denatured material (H.BSA) were similar. These enzyme values represent the enzyme activity of the intact large

granular fraction. From this evidence the postulation of enzyme changes must be related to a cell type other than the macrophage. In order to make this assumption several experiments must be considered. The first piece of supporting evidence for the lymphocyte participation in these enzyme changes comes from the gradient studies involving the liver and spleen. Figures 5 and 6 reveal that in the liver there are no differences in the enzyme profile when H.BSA and the normal, non-stimulated controls are compared. Since the liver is predominantly a phagocytic organ two possible explanations for this observation, that is the similar enzyme levels, follows: first, the phagocytic cells may have ingested the material, degraded it and returned to their normal acid phosphatase level or secondly, the phagocytic cells were not involved, exclusively, in the removal of the foreign material. If the spleen results are examined (see figures 9 and 10) and the same arguments are applied, then the depression noted following the injection of H.BSA does not apply. If the macrophages had engulfed the material, degraded it and returned to their normal enzyme level, then the reasons for the depression noted in the spleen, must involve another cell type, a cell type other than the macrophage. It is proposed that the enzyme changes occur through lymphocyte involvement. As will be discussed later in this chapter, other experiments further point to the lymphocytes and their participation in the observed enzyme changes. Furthermore, the depression in enzyme activity follows after the administration of only the immunogenic materials, rather than after the injection of a material which has been shown to induce tolerance. These differences are significant due to the non-overlapping of the various experimental results. In the case of haemocyanin as well as heat-denatured BSA,

the depression occurs within 48 hours of antigenic stimulation.

Allison and Mallucci (1964) have shown that in vitro tissue cultures of human peripheral blood lymphocytes when stimulated with phytohaemagglutinin (PHA) increases their lysosome number, as judged by histochemical means, during cellular transformation and that prior to cell mitosis there is a loss of these cytoplasmic organelles. This has been shown by Hirschhorn and her co-workers in a separate study. The phytohaemagglutinin stimulation of human lymphocytes was shown to lead to a rise in the acid phosphatase granules during the mitosis of the cells. These increases were mainly determined by histochemical means. If the increases were assessed biochemically, they were not as profound but nonetheless, increases were observed.

The density distribution of the enzyme activity also supports the postulation of lymphocyte involvement with respect to the cell's possession of lysosomes. Bowers and de Duve (1967) were able to separate the LG fraction of the rat spleen homogenate into two separate density regions: 1.15 and 1.19. The 1.15 group were incomplete in their enzyme make-up whereas the 1.19 group contained the full complement of hydrolytic enzymes. The latter group has been associated with the macrophages since these cells are constantly involved in engulfment and breakdown of foreign as well as "self" material and therefore would need the full range of destructive enzymes while the 1.15 group has been assigned to the lymphocytes. Bowers (1969) suggests that these 1.15 lysosomes may have an immunological function in that since they are incomplete with respect to the enzyme content, perhaps they are only capable of partial degradation of ingested material and therefore they would preserve the antigenic intregrity of the material.

The findings of the density gradient studies, in this present

investigation, reveal that the maximum enzyme activity was to be found in the density region of 1.100 to 1.125. There were no peak activities observed in the 1.19 density region of the homogenates tested (see figure 8). Therefore these results further support the view that it is the lymphocyte lysosomes which are being assayed and which are responsible for the enzyme changes observed.

Lysosomal enzyme changes over a period of time, after antigenic stimulation

The study of intact lysosomes of the LG fraction has revealed that over the first 48 hours after antigenic stimulation there follows enzyme changes which, by the 8th day, have returned to normal enzyme levels (see figures 12 and 13). Similar findings were found in a recent paper by Lozzio and his co-workers (1969) in which the hydrolytic enzyme changes were observed over time after the injection of phytohaemagglutinin in mice. There was an initial decrease in the amounts of acid phosphatase detected in the spleen, but by the 9th day after the injection of PHA the enzyme levels had returned to the normal, pre-injection level. The reduction in acid phosphatase activity, as noted by Lozzio and his group, amounted to approximately 50%; this figure is in agreement with the results obtained in the present study. Figure 6 which represents the enzyme changes after the administration of H.BSA also shows a 50% reduction in enzyme activity as compared to the normal, non-stimulated control (see figure 5).

The induction of tolerance in CBA mice

The effect of the physical state of BSA upon the immune response to BSA in adult mice has been studied. The physical state of the antigen has been shown to have a marked effect on the immune response. In this present study, it was found that 0.05 mg C.BSA was capable of tolerance induction whereas if the same quantity of heat-denatured material (0.05 mg) were administered, there was less probability of tolerance becoming established.

The heat-denaturation of BSA and centrifuged, aggregate-free form of the antigen have been shown to behave differently. Freeman (1959) has shown that heat-denatured albumins are cleared at a faster rate than are the native albumins. Earlier, Cooper and Neurath (1943) examined the effects of heating on horse serum albumin. They found that particle size and shape, electrophoretic mobility and pattern as well as susceptibility to trypsin digestion were different to the native protein.

Biro and Garcia (1965) were able to induce tolerance in adult rabbits after the injection of 5 mg centrifuged, aggregate-free HGG; heat-aggregated HGG, if given in the same quantity, was a powerful immunogen. This work demonstrated that centrifugation of the material removed potentially antigenic molecules of the antigen. Heating of the material produced an immunogen with a different physical state brought about by the denaturation process.

Many investigators have been able to induce tolerance with aggregate-free material. As reviewed in the introduction, Dresser (1962), first reported the induction of tolerance using an aggregate-free preparation of BGG. The use of BSA, in its various antigenic forms, has been extensively studied. Due to the familiarity in this research unit of BSA and its tolerance inducing abilities, this line

of research was continued. Pinckard (1967) was able to show that the degree of aggregation of BSA was of prime importance in the induction of tolerance in adult rabbits. It was found that if C.BSA were administered, tolerance would be induced, whereas if the same quantity of NBSA were injected, a hyporesponsive state resulted. The injection of alum-precipitated BSA, a highly particulate form of the antigen, resulted in a powerful response in rabbits.

The present study was able to make use of this experience by first inducing tolerance in mice, following a single, intraperitoneal injection of the appropriate antigenic form, and secondly, by following the enzymatic changes which accompanied the injection of the respective forms of BSA.

The localization of isotope-labelled C.BSA or H.BSA in the liver and spleen of CBA mice

The injection of radioactively-labelled antigen is a means of studying the associations of antigen in its travels through the body fluids and tissues. Upon injection of I^{125}-labelled C.BSA it was found that when compared with I^{125}-labelled H.BSA there was no significant difference in the uptake of the antigens in the organs examined (see Table VI). This point is of prime importance when comparing the observed enzyme changes, for it reveals that the quantity of antigen present in the organs is the same. Therefore the increase in enzyme levels after the administration of C.BSA and the decrease in levels after H.BSA may not be due to differences in antigen concentration in the organs. It is for this reason that the enzyme changes are thought to be due to the physical nature of the antigen.

In the case of the C.BSA localization, the soluble, aggregate-free antigen localized in a general, diffuse pattern. Heavily labelled concentrations appearing in the arterioles indicate a large quantity

of label still present in the circulation. Some of the macrophages, in the red pulp, had taken up the material, but the general pattern was found to be diffuse. The localization patterns, as determined by serial section examination of the tissue revealed specific localization patterns. When the organs treated with H.BSA were examined, there was found to be heavy labelling in the red pulp with the tingible body (dendritic) macrophages involved in the uptake of the antigen. The marginal areas of the malpighian follicles (also referred to as germinal centres) were becoming labelled and in general the localization patterns of the denatured antigen were more concentrated within the phagocytic cells than that observed when the antigenic material was the soluble, aggregate-free form of BSA. In the liver there was little uptake by the Kupffer cells and the predominant featureof the localization patterns was one of diffuse grain localization rather than specific uptake by either the phagocytic cells or the parenchymal cells.

The results obtained in the spleen are very much in agreement with the results of other investigators. Ada and Lang (1966) using I^{131}-labelled flagellin, which is the soluble form of the antigen, found that although dendritic macrophage participation in the uptake of the antigen did occur, there was also a general diffuse pattern with the soluble antigen although eventually the antigen did localize within the medullary macrophages of the lymph nodes and in the tingible body macrophages of the spleen red pulp and marginal zones.

Thus the localization patterns of the two antigenic forms follow similar uptake distributions and these results are similar to those of other workers.

The column separation of splenic lymphocytes after antigenic stimulation

It has been shown that upon the injection of two different, physical forms of BSA there follows opposing enzyme changes in the spleen of CBA mice. The column separation studies enable the separation of the splenic lymphocytes on the basis of the chemical changes occurring at the cell surface. Plotz and Talal (1967) were able to show that the cells obtained initially from the column were normal, non-sensitized cells in that they were unable to form rosettes when mixed with sheep erythrocytes. However, the cells eluted from the column towards the end of the separation procedure were able to form rosettes when sheep red blood cells were added.

The results of the separation trials (see figure 15) reveal that a similar distribution of cells occurred. In order to show that the cells obtained in the 5th fraction were sensitized lymphocytes, a pilot study was attempted whereby the animals were injected with 2×10^8 sheep erythrocytes. Figure 14 provided further evidence that the 5th fraction cells were in a state of producing anti-sheep erythrocyte antibody, in that these cells contained 33% rosette-forming cells whereas the cells obtained from the first fraction in the separation procedure contained only 1% rosette-forming cells.

Upon analysis of the 1st and 5th fraction cells for the acid phosphatase content, it was found that in all of the cases examined, there was an increase in the amounts of acid phosphatase (see Table VII) in the 5th fraction cells, after the administration of either antigen, when compared to the non-stimulated control. These results thus correlate with both the spleen cell studies (see figure 2) and the disrupted LG fraction studies (see figure 3) in that upon stimulation with either the denatured antigen or the soluble material there followed an increase in the total acid phosphatase content of the organ.

The results of these separation studies implicate the lymphocyte as being part of this enzyme change which has been observed. The fact that the purified lymphocyte preparations, after passage through the leucopac columns, still retained their enzyme activity illustrates that while the macrophage donates enzyme activity as noted above, the lymphocyte, nonetheless, participates in the total activity observed. The results of these studies are similar to those attempted earlier, in that both physical antigenic forms of BSA induced higher enzyme levels than those which occurred in the controls (see figure 2). The use of pure lymphocyte preparations therefore adds further strength to the postulation of lymphocyte inducement in the observed enzyme changes.

Histochemical studies involving purified lymphocyte preparations

Little is known about the lysosomal population of the lymphocytes and many workers in the field of immunology to-day still dispute their presence. It has been shown that density gradient centrifugation of the lysosomal populations can resolve two distinct groups perhaps three. As reviewed earlier, this feature of lysosomes has been demonstrated by Bowers and de Duve (1967). Several investigators have been able to show that lymphocytes, usually human peripheral blood cells, if stimulated in vitro with PHA would increase their total number of lysosomes as indicated by the acid phosphatase stain (Allison and Mallucci, 1964; Hirschhorn, et al, 1964; Hirschhorn, et al, 1965).

In the present study, histochemical staining techniques were employed after the administration of the two forms of BSA; C.BSA and H.BSA. In each of these test systems, there followed an increase in the number of detectable acid phosphatase positive grains.

Conclusions

From the results outlined in the previous chapter and the review of these results and their possible implications the following conclusions may be drawn from this present study.

The administration of a soluble, aggregate-free form of BSA (C.BSA) was found capable of tolerance induction, at the same time, there was an overall increase in the detectable quantities of acid phosphatase in the intact lysosome preparation. If the same quantity of heat-denatured material were administered, the induction of tolerance was not as profound and the accompanying enzyme assay revealed a depression in the enzyme activity of the intact lysosome preparation. This important fact, combined with the similar results obtained in the case of the haemocyanin studies, suggests that the labilization effect of the tolerogenic antigen and the stabilization effect of prospective immunogens hold an important key in the distinction between the two classes of foreign, antigenic material. In the case of all antigenic stimuli, when the large granular fraction had been disrupted, there was an increase above the control values; when the large granular fraction was not disrupted, there were the observed differences in the antigenic forms and the detectable amounts of acid phosphatase.

From the results discussed above it would appear, that the physical state of the antigen brings about either the labilization of the lysosomal membrane in the case of the tolerogenic antigen and stabilization of the lysosomal membrane, in the case of the immunogenic antigen. The significance of these findings with respect to the early stages of the immune response or in inductive phase of tolerance will be discussed in the following section on the theoretical aspects of antibody production and the induction of tolerance.

Further experiments are required with other tolerogenic antigens in order to compare the enzyme changes presented in this work with those which will be calculated with future studies. The antigenic form used for tolerance induction in this study was BSA which is a relatively pure as well as simple protein material; the use of more complex antigens must also be studied and their enzyme changes must be determined.

As discussed earlier in this work, the labilization and stabilization of the lysosomal membranes with respect to pathological conditions has been described by Weissmann (1967). Thus if the labilization of lysosomes occurs after the administration of a tolerogenic antigen, the administration of a stabilizing agent may bring about the initiation of antibody production rather than the induction of tolerance. Similarly, if a labilizing agent were administered along with the injection of an immunogenic material, the results could possibly be the abolition of the immune response and the induction of tolerance.

THEORETICAL ASPECTS OF THE MECHANISMS OF ANTIBODY FORMATION AND THE INDUCTION OF TOLERANCE IN LIGHT OF THE PRESENT FINDINGS

Mechanisms of antibody formation

The mechanisms of antibody formation have for a long time been debated, reviewed and discussed. Many theories have been put forth in the last 50 years, yet the problem still remains and the mystery of the mechanism of an antibody response to a specific antigen remains unsolved. At present there are at least two schools of thought as to the mechanisms of the immune response: 1) The instructive theory instituted by Breinl and Haurowitz (1930), Mudd (1932) and Alexander (1932) and 2) The selective theory initiated by Burnet and Fenner (1949) and Jerne (1955). The complexity of the arguments put forth by these two schools of thought are now the concern of the molecular biologists and animal geneticists. The mechanisms discussed and which are relevant to the subject matter of this thesis will deal with the mode of action of the antigen in the inductive phase of the immune response.

The instructive theories of antibody synthesis although attractive are poorly represented by experimental evidence. Pauling (1940) expressed his classical modification of the Haurowitz-Mudd-Alexander view by proposing that antibody differs from one type to the next only ".... in the way in which the two end parts of the globulin polypeptide chains are coiled, these parts ... having accessible a very great many configurations with nearly the same stability; under the influence of an antigen molecule, they assume configurations complementary to surface regions on the antigen, thus forming two active ends". In Pauling's paper there is no mention of the process by which this intramolecular three-dimensional folding could be stabilized.

To a large degree, the instructive theory is taken from an analogy with other biological processes, the induction of enzyme synthesis being only one example.

The most popular theory of antibody production is that of the clonal theory, initially proposed by Burnet and Fenner (1949), which was not established from direct studies on the synthesis of antibody but from the collected data as a means of explaining immunological tolerance. This theory postulated genetically, pre-determined "clones of cells" which were reactive for every form of antigen; antigen affecting the surface of the cells would trigger off transformation and proliferation of the cell line. If these pre-determined clones were eliminated during the embryonic development of the animal by a "self" recognition mechanism, the induction of tolerance could easily be established. The main objection to this theory is the multitude of antigenic determinants which would be required by the cells. This objection has been partially answered in the clonal selection theory of Burnet (1959) which eliminated the necessity of pre-determined clones. However, problems immediately arose, Humphrey (1964) found that rabbits rendered tolerant to BSA during neonatal life did not recover their reactivity to the antigen for many months. This would suggest that the mutation rate of the rabbits must be very low (Humphrey, 1965). From the clonal selection theory the shift has been towards a "multipotent clonal" theory. Support for this theory stems from the studies of Kim, Bradley and Watson (1966). Their antigen competition studies demonstrated that neonatal piglets, which were immunologically competent at birth, were highly susceptible to antigenic competition even through dietary antigens. Austin and Nossal (1966) found that the induction of tolerance in neonatal rats to one particular flagellar antigen inhibited the response upon later

challenge with other chemically unrelated flagellar antigens. Felton and Mekori (1966) provided direct evidence for the multipotent clonal theory by demonstrating that "cloned-cell" populations of lymphocytes were pluripotent.

The studies of Fishman (1961) suggested a cooperation between the macrophage and the lymphocyte in the induction of the antibody response. Several investigators have shown that the macrophage does not participate in the processing of all antigens prior to the synthesis of antibody (Boak, Kölsch and Mitchison, 1968). Thus upon stimulation either through the macrophage or by some other means, the lymphocyte synthesizes antibody which is specific for the appropriate antigen. This stimulation at the cell surface may involve a moiety donated by the macrophage (Fishman, 1961) and the transformation of the cell which follows may involve the lysosomal enzymes in the triggering mechanism. At this stage in the proceedings there follows a stabilization of the lysosomal membranes thus ensuring the prolongation of the cell itself. From the results presented in this work, the immunogenic antigens brought about a depression in the enzyme levels of the spleen after the injection of the material in question and these enzyme levels remained suppressed, as compared to the normal, non-stimulated control animals, returning to the normal levels after 8 days. If indeed, antigens stimulated the formation of lysosomes in previously "naive" lymphocytes, the induction of these organelle, which are rich in potentially injurious substances, may be one means whereby tissue injury is effected in the immune response for Dumonde (1965) has shown that antibody against Ascites tumor cells can result in increased lysosome permeability. The lysosomes and their involvement in the immune response must be, at this stage of our knowledge, of a speculative nature. However, future investigations may prove this hypothesis correct. The injection of known labilizing

agents, such as vitamin A, may upon administration with the immunogenic material negate the immune response to the antigen and induce a tolerant state. Similarly, other labilizing agents must be investigated and their effect on the immune response studied.

Mechanisms of tolerance induction

In any actively developing field of inquiry, theories will come and theories will go. Their utility can be measured by the amount of investigation they have engendered in response to the direct propositions they present.

The theory presented in this work, on the induction of tolerance, is not a novel one and has already appeared in several variations in the literature (Dresser, 1962; Mitchison, 1964; Frei, Benacceraf and Thorbecke, 1965; Nossal and Austin, 1966). Accepting minor variations in the selective mechanisms of the immune response (see Introduction/ Mechanisms of antibody production), one may propose the existence of a population of cells capable of reacting with a suitable antigenic determinant. The origin of these cells is not especially pertinent here. These cells have on their surface or possibly in their surface an antibody-like receptor which is capable of combining with specific antigenic determinants. There may be one type of specific site per cell (clonal selection theory) or there may be many different kinds of sites located on each cell (subcellular selection theory) either of these may be applicable to this hypothetical model. However the full activation of this cell requires the combination of the antigenic determinant with its specific site on the cell plus the addition of another moiety which will be contributed by the macrophage. If the macrophage moiety is lacking during this combination, it follows that there will be a direct binding of the combining site of the cell with the antigenic determinant on the antigen. This action will block the

cell from future combinations with the complexed antigen in the presence of the macrophage moiety and the result will be an inhibited or tolerant cell. In the past there was no evidence to suggest whether this inappropriate combination of direct antigen binding to the cell surface destroys the cells or merely renders them inactive to further antigenic stimulation. At this point it should be noted that the results of this present study reveal a labilization of the lysosomal membrane upon the administration of a tolerogenic material. For this reason it is proposed that through the direct action of the antigen, in combining with the cell, there follows a labilization of the lysosomal membrane which eventually results in the self destruction of the cell itself.

Makela and Mitchison (1965) demonstrated, by means of the adoptive transfer of sensitized spleen cells to unsensitized, X-irradiated, isogenic mice, that tolerance could be induced in the recipient animals upon the administration of high doses of antigen; if, on the other hand, the sensitized cells were incubated in vitro for 6 to 24 hours with the antigen, were washed and injected into the recipients, an enhanced antibody response occurred rather than the induction of tolerance. From this work comes the importance of the in vivo environment in the induction of an unresponsive state which could perhaps involve the presence of complement.

An analogy from the literature may explain the proposed hypothesis. Dumonde and his associates (1965) allowed antibody directed against whole ascites tumor cells to interact in the absence of complement. The antibody did not enter the cell and intense lysosomal activation followed. It was found that labilization of the lysosomal membranes of the tumor cells followed with the ultimate destruction of the cell itself if complement were present. Antigen coming into contact with the lymphocyte directly may in the presence of complement bring about

the labilization of the lysosomal membranes and the subsequent self-destruction of the cell. Thus tolerance induction possibly involves the simple mechanism of clone-depletion. Since younger animals recover from tolerance more rapidly than older animals (Mitchison, 1965), the greater turnover of cells, in the former group, allows for new clones of cells to become established. This hypothesis although partially proven, in the case of BSA and tolerance induction, requires further experimentation employing other antigens. The use of stabilizing agents may shed additional light on the underlying mechanisms on the induction of immunological tolerance. Our knowledge, since the days of Metchnikoff, has advanced and in the coming years many of the immunological problems of to-day may be unravelled through the aid of the molecular biologists and their biochemical understanding.

> "Let the enzymes speak, listen to them, and they will tell you a great deal: about the size and shape of their intracellular locus, about the envelopes which shroud them, about their companions, and eventually, if it was not known before, about their role in the machinery of life ..." (de Duve, 1963).

SUMMARY

SUMMARY

The work reported in this thesis has shown that the physical state of the antigen has a marked effect on the lysosomal enzyme levels of the spleen. CBA mice were injected with two different physical forms of bovine serum albumin (BSA). One form was of a particulate nature whilst the other was in the form of an aggregate-free material. The particulate form of the antigen was found to be more immunogenic than the soluble, aggregate-free material, which was found capable of tolerance induction.

Upon administration of these two antigenic forms, there followed an increase in the acid phosphatase levels of the spleen. The testing of either 10^6 spleen cells or the large granular fraction, which has been shown to contain mitochondria, microsomes, cell membrane fragments as well as lysosomes, of the spleen yielded similar results. Both test systems when compared with the control animals resulted in an increased acid phosphatase enzyme level. In each case the mixtures assayed represented the acid phosphatase levels of disrupted lysosomes. Intact lysosomes, on the other hand, revealed that the soluble, aggregate-free form of the antigen (C.BSA) caused a similar increase in the amount of acid phosphatase detected as compared to the disrupted organelles after antigenic stimulation. The particulate, heat-denatured BSA, in contrast, gave a marked depression in the amount of acid phosphatase present, as compared to the controls. Similarly, the injection of haemocyanin, which is also immunogenic; as is the particulate BSA, resulted in a depression in the amount of acid phosphatase levels forty-eight hours after antigenic stimulation.

The lymphocyte appears to be one of the cell types which is implicated in these enzyme changes although the role of other cells has still to be established. Column separation of splenic lymphocytes as well as histochemical staining of "purified" lymphocytes suspensions revealed increase acid phosphatase levels in the cells after antigenic stimulation.

Autoradiographic studies suggest that the aggregate-free form of the antigen is not localized to the same extent as is the particulate form of BSA. The determination of the total quantity of the respective isotope-labelled antigen, present in the organs examined, reveals that there is no significant difference in the amount of antigen present whether the material be in the soluble or particulate form. The enzyme changes observed must therefore be due to the physical state of the antigen and its effect on the cell types involved and not to the quantity of antigen present at the time of the enzyme determinations.

It is postulated that since both forms of the antigen are in equal proportions, as indicated by the counts/mg tissue, in the organs examined, the changes in the enzyme levels observed may be a reflection of mechanisms of the immune process and the induction of tolerance after the administration of various physical forms of the antigen.

The involvement of the lysosomes in the induction of tolerance is discussed; the immunogenic material and its effect on the lysosomal enzymes is also discussed.

APPENDIX

APPENDIX

Acetate Buffer, 0.4M, pH 5.0

Stock solution A: 0.4M acetic acid (23.0 ml. in 1000.0 ml. distilled water)

Stock solution B: 0.4M sodium acetate (32.8 g $C_2H_3O_2Na$ or 54.4 g $C_2H_3O_2Na;3H_2O$ in 1000.0 ml. distilled water)

Final stock solution of pH 5.0:

Mix 14.8 ml. solution A + 35.2 ml. solution B, bringing the final volume to 100.0 ml.

Borate Buffer, pH 8.4 (ionic strength 0.1)

boric acid	6.184 g
sodium tetraborate	9.536 g
sodium chloride	4.384 g

Made up to 1000.0 ml. with distilled water.

Hank's solution

solution A

NaCl	160 g
KCl	8 g
$MgSO_4 \cdot 7H_2O$	4 g
deionized water	800 ml
CaCl	2.8 g
deionized water	100.0 ml

Mix slowly and bring to 1000.0 ml.

solution B

$Na_2HPO_4 \cdot 12H_2O$	3.04 g
KH_2PO_4	1.2 g
glucose	20.0 g
water	800.0 ml.

when dissolved, add 100.0 ml. 0.4% phenol red in NaOH. Adjust volume with deionized water, to 1000.0 ml. add 2.0 ml. chloroform and store at 4° C.

Hank's solution, continued

Mix 100.0 ml. solution A and 100.0 ml. solution B, adding 1800 ml. deionized water. The solutions were autoclaved at 10 lbs. for 10 minutes.

Note:

Hank's solution without Ca^{++} and Mg^{++} was prepared in the same manner as described above, except that the Ca^{++} and Mg^{++} ions were omitted.

Phosphate buffered saline (pH 7.2)

NaCl	8.00 g/litre
K_2HPO_4	1.21 g/litre
KH_2PO_4	0.34 g/litre

The chemicals were dissolved and the final volume was made up to 1000.0 ml.

Appendix Cont'd).

Rank Sum Test

The purpose of the rank sum test is to compare two unmatched random samples of measurement. The usual assumption is made that there is no significant difference between the two sets of measurements with respect to the procedures carried out on the samples. Such tests are called "distribution-free".

The data was pooled and rearranged in order of size. This was called ranking the pooled measurements. Each measurement was accorded a rank value number according to merit (i.e. the largest to the smallest, or vice versa). If two numbers possess the same measurement they are given the same rank value ($x_1 + x_2 + \ldots /n$). The assigned ranks were totalled (for each individual group) and the resulting sum was correlated with the Rank Sum Statistical Tables. If the number (rank total) was less than or equal to the table number, the sample was then significant to the degree stated.

The following example taken from this text illustrates the nature of the Rank Sum Test: Data from Table I Haemocyanin vs Control.

Ranked Data:

0.12	0.2	0.2	0.29	0.3	0.38	0.54	0.6	0.76	0.81
C	C	C	H	H	C	H	H	H	H
1	$2\frac{1}{2}$	$2\frac{1}{2}$	4	5	6	7	8	9	10

C total = 12 $(1+2\frac{1}{2}+2\frac{1}{2}+6)$

H total = 43 $(4+5+7+8+9+10)$

From the statistical tables it was found that the results were significant at the 5% level.

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